COMPARATIVE EVALUATION OF CPP-ACFP AND FUNCTIONALISED TRICALCIUM PHOSPHATE (fTCP) PASTES ON REMINERALIZATION AND EROSION RESISTANCE OF REMINERALIZED TOOTH ENAMEL - AN IN VITRO STUDY

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in partial fulfilment of the requirements

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BRANCH – IV

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CERTIFICATE

This is to certify that **Dr. M.RAMESH ,** Post Graduate student (2010 - 2013) in the Department of Conservative Dentistry and Endodontics, has done this dissertation titled "**Comparative evaluation of CPP-ACFP and functionalised tricalcium phosphate (fTCP) pastes on remineralization and erosion resistance of remineralized tooth enamel-An in Vitro Study**" under my direct guidance and supervision in partial fulfilment of the regulations laid down by **The Tamilnadu Dr. M.G.R. Medical University, Guindy, Chennai – 32** for **M.D.S.** in Conservative Dentistry and Endodontics (Branch IV) Degree Examination.

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DECLARATION

TITLE OF DISSERTATION	Comparative evaluation of CPP-ACFP and functionalised tricalcium phosphate (f TCP) pastes on remineralization and erosion resistance of remineralized tooth enamel -An in vitro study
PLACE OF THE STUDY	Tamil Nadu Government Dental College & Hospital, Chennai – 3.
DURATION OF THE COURSE	3 YEARS
NAME OF THE GUIDE	DR. S.JAIKAILASH
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I hereby declare that no part of dissertation will be utilized for gaining financial assistance or any promotion without obtaining prior permission of the Principal, Tamil Nadu Government Dental College & Hospital, Chennai – 3. In addition I declare that no part of this work will be published either in print or in electronic media without the guide who has been actively involved in dissertation. The author has the right to preserve for publish of the work solely with the prior permission of Principal,Tamil Nadu Government Dental College &Hospital,Chennai -3.

HOD **GUIDE** **Signature of the Candidate**

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This agreement herein after the "Agreement" is entered into on this day **Dec 2012** between the Tamil Nadu Government Dental College and Hospital represented by its **Principal** having address at Tamil Nadu Government Dental College and Hospital, Chennai - 600 003, (hereafter referred to as, 'the college')

And

MR.Dr.S.JAIKAILASH aged 38 years, working as **Professor** in Department of Conservtive Dentistry & Endodontics at the college, having residence address at T1, Vinoth Vetri Apartments, 37,Govindan Street, Ayavoo Colony, Aminjikarai, Chennai - 29 (herein after referred to as the 'Principal Investigator')

And

Mr.Dr.M.Ramesh aged 29 years currently studying as **Post Graduate student** in Department of Conservtive Dentistry & Endodontics, Tamilnadu Government Dental College and Hospital, Chennai - 3 (herein after referred to as the 'PG student and co-investigator').

Whereas the PG student as part of his curriculum undertakes to research on **COMPARATIVE EVALUATION OF CPP-ACFP AND FUNCTIONALISED TRICALCIUM PHOSPHATE (fTCP) PASTES ON REMINERALIZATION AND EROSION RESISTANCE OF REMINERALIZED TOOTH ENAMEL-AN IN VITRO STUDY** for which purpose the Principal Investigator shall act as principal investigator and the college shall provide the requisite infrastructure based on availability and also provide facility to the PG student as to the extent possible as a Co-investigator

Whereas the parties, by this agreement have mutually agreed to the various issues including in particular the copyright and confidentiality issues that arise in this regard.

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8. The Principal Investigator shall suitably guide the Student Research right from selection of the Research Topic and Area till its completion. However the selection and conduct of research, topic and area of research by the student researcher under guidance from the Principal Investigator shall be subject to the prior approval, recommendations and comments of the Ethical Committee of the College constituted for this purpose.

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In witness where of the parties herein above mentioned have on this the day month and year herein above mentioned set their hands to this agreement in the presence of the following two witnesses.

College represented by its **Principal** **PG Student**

Witnesses

1.

Student Guide

2.

ABSTRACT

Aim:

To compare and evaluate the effects of **CPP-ACFP** and **functionalised tricalcium phosphate** (fTCP) pastes on remineralization and erosion resistance of remineralized tooth enamel.

Materials and Methods:

From 15 unerupted, matured human third molar teeth, 60 enamel slabs were made.Out of these,45 enamel slabs were selected with microhardness values in a range of 340±10 VHN and followed by SEM analysis.After this,enamel slabs were immersed in cola drink for 8 minutes followed by microhardness and SEM analysis.Then softened enamel slabs were randomly divided into 3 groups as follows:Group I-Control (n=15),Group II- CPP-ACFP (n=15),Group III – fTCP (n=15).Group I received no treatment and was stored in artificial saliva for 36 hrs while Group II and Group III were treated with CPP-ACFP and fTCP pastes respectively for 3 min at 0, 8, 24, 36 hrs.All enamel slabs were taken for assessment of surface microhardness and SEM analysis.Acid challenge of remineralized enamel slabs were carried out by immersing in cola drink for 8 min followed by microhardness and SEM evaluation.Statistical analysis was performed using one way ANOVA and Tukey’s HSD Post-hoc test at a significance level of 0.05.

Results:

Results of the microhardness measurement showed that Group III (fTCP) exihibited statistically higher remineralizing potential than Group II (CPP-ACFP) and Group I (Control).Results of the SEM analysis showed that Group III (fTCP) resulted in enhanced remineralization throughout the surfaces, with a more homogenous morphology while Group II (CPP-ACFP) resulted in adequate remineralization with reduction in porosities and Group I (control) resulted in inadequate remineralization.

Conclusion:

Within the limitations of this in vitro study, fTCP showed higher remineralizing potential and acid resistance compared to CPP-ACFP.

Key words: Erosion, Remineralization, Demineralization, CPP-ACFP, fTCP.

CONTENTS

LIST OF TABLES

LIST OF GRAPHS

INTRODUCTION

Erosive tooth wear or dental erosion is defined as a localized loss of the tooth surface by a process of acidic dissolution without the involvement of bacteria[46].Dental erosion is a growing problem especially among young people[75]. In contrast to dental caries, which is usually localised, erosion is often generalised[30]. Acids which cause dental erosion may be intrinsic or extrinsic in origin.Dietary acids are the most widely studied aetiological agents, said to be the most important extrinsic factor[27].

The prevalence of erosion is increasing nowadays, reflecting the wwide availability and frequent consumption of acidic and carbonated beverages, fruit juices, wines, and sport drinks[10].The severity of erosion in the oral environment is related to the effectiveness of the protective mechanisms which includes composition of saliva, flow rate, buffering capacity and individual dental anatomy. In the oral environment, host factors (such as, plaque, pellicle formation and mineral concentration of the tooth) can influence the progression of demineralization phenomenon. Salivary factors such as the salivary flow rate, buffering capacity, and composition might exert protective action on dental surfaces.

It is important to search for remineralizing agents to prevent or repair dental erosions[45], the beneficial effects of fluoride arise from its incorporation as fluorapatite in tooth substances leading to the decreased solubility of tooth enamel.Fluoride releasing materials may act as reservoir of fluoride and may increase the fluoride level in plaque, saliva, and the dental hard tissues. Casein phosphopeptide -amorphous calcium phosphate complex (CPP–ACP) has been introduced commercially as a source of calcium and phosphate ions in the oral environment. CPP–ACP has been successfully incorporated into oral health care products as sugar-free chewing gums, mouthrinses, and sports drinks to reduce dental erosion[1].

Casein phosphopeptides (CPP) bind to calcium and phosphates ions in nanoparticles, preventing their crystals from growing to critical size and precipitating out of the solution.The amorphous calcium phosphate is able to release calcium and phosphate ions from its biologically active state to maintain the supersaturated state, thus enhancing the remineralization process. CPP stabilizes ACP phase in which fluoride ion had been incorporated to produce a novel amorphous calcium fluoride phosphate phase (ACFP) on the tooth surface. The identification of amorphous calcium fluoride phosphate (ACFP) from ACP and fluoride led to the observed additive effect of CPP-ACP and fluoride[7] from the formation of this phase.

CPP-ACP with incorporated fluoride (CPP-ACFP) of 0.2%, (900ppm), which approximates that of adult strength toothpastes and has greater remineralizing potential effect on carious lesions compared with those of fluoride alone.The remineralization effect of CPP–ACP with 900 ppm of fluoride, was found to be superior to that of CPP–ACP alone in an in-situ study[72].

Tri-Calcium Phosphate (TCP), a proprietary calcium phosphate ingredient which has been recently introduced can be protected from unwanted interactions with fluoride during storage. In addition, this innovative technology in toothpaste can be tailored to provide short- or long-term mineral delivery in a variety of dental product. Synergistic combination of the functionalized calcium phosphate with NaF provides greater remineralization in terms of surface microhardness and enamel fluoride uptake relative to fluoride alone[34].There are only limited comparative studies with regard to functionalized tricalcium phosphate paste.

Softening of the enamel surface is an early manifestation of the erosion process. Reduced surface hardness which accompanies erosion of the enamel surface by acidic beverages can be assessed using a physical quantitative measurement such as the hardness test or qualitative measurement such as Scanning Electron Microscopy (SEM). Microhardness indentation provides a relatively simple, nondestructive and rapid method for classification of materials and for comparative studies of their properties[24] and have been employed in the study of' remineralization phenomena.

The purpose of this study was to evaluate the effects of CPP–ACFP and functionalised tricalcium phosphate (fTCP) pastes on remineralization and erosion resistance of remineralized tooth enamel using Microhardness and SEM analysis.

AIM AND OBJECTIVES

Aim:

To compare the remineralizing potential of **CPP-ACFP** and **functionalised tricalcium phosphate** (fTCP) pastes on remineralization and erosion resistance of remineralized tooth enamel.

Objectives:

1. To compare the remineralizing potential of CPP-ACFP and functionalised tricalcium phosphate (fTCP) on demineralized enamel slab.
2. To compare the erosive resistance potential of CPP-ACFP and functionalised tri calcium phosphate (fTCP) pastes on remineralized enamel slab.

   By using

i) Surface micro hardness measurement (SMH) and

ii) Scanning electron microscopy (SEM)

CPP ACP and CPP-ACFP

CPP-ACP is the acronym for a complex of casein phosphopeptides and amorphous calcium phosphate.Casein phosphopeptides (CPP) are a group of peptides derived from casein, part of the protein that occurs naturally in milk.The CPP are thought to be responsible for the high bioavailability of calcium from milk and other dairy products.

Reynolds and Johnson (1981)[64] found that supplementation of a cariogenic diet with cow's milk reduced substantially dental caries incidence and importantly this was not due to reduced consumption of the cariogenic diet.

Reynolds et al (1987)[59] determined the ability of casein and found that casein and tryptic peptides to prevent enamel demineralization was related to their incorporation into plaque, thereby increasing plaque calcium phosphate and acid-buffering capacity by the phosphoseryl, histidyl, glutamyl, and aspartyl residues and indirectly through catabolism by plaque bacteria.

Reynolds et al (1995)[63] stated that casein phosphopeptides (CPP) are produced from a tryptic digest of the milk protein casein by aggregation with calcium phosphate and purification by ultrafiltration. The CPP have a remarkable ability to stabilise calcium phosphate in solution and substantially increase the level of calcium phosphate in dental plaque. Through their multiple phosphoseryl residues the CPP bind to clusters of amorphous calcium phosphate (ACP) in metastable solution, preventing their growth to the critical size required for nucleation and precipitation.

Roberts et al (2000)[67] determined the ability of CPP-ACFP to remineralize fluorotic lesions and improve the appearance of teeth and found that treatment with CPP-ACFP significantly reduced the whiteness and partially remineralised fluorotic lesions. Surface conditioning improved the effectiveness of the treatment with CPP-ACFP.

Shen et al (2001)[69] studied the ability of CPP-ACP in sugar-free chewing gum either sorbitol- or xylitol to remineralize enamel subsurface lesions in a human in situ model. They found that the addition of CPP-ACP to either sorbitol- or xylitol-based gum resulted in a dose-related increase in enamel remineralization.

Shen et al (2004)[70] compared the enamel remineralization ability of mouthrinse containing CPP-ACP with that of a mouthrinse containing CPP-ACP and 220 ppm F. and mouthrinse containing neither CPP-ACP nor fluoride (placebo).The mouthrinse containing CPP-ACP with 220 ppm F- produced maximal enamel subsurface lesion remineralization followed by CPP-ACP and placebo.These results support the role of fluoride in promoting remineralization and demonstrate an important facilitation of the effect of fluoride by CPP-ACP.

Iijima Y et al (2004)[29] investigated the acid resistance of enamel lesions remineralized in situ by a sugar-free chewing gum containing casein phosphopeptide-amorphous calcium phosphate nanocomplexes (CPP-ACP: Recaldent) using microradiography The acid challenge after in situ remineralization for both control and CPP-ACP-treated lesions resulted in demineralization underneath the remineralized zone, indicating that the remineralized mineral was more resistant to subsequent acid challenge. The results showed that sugar-free gum containing CPP-ACP is superior to an equivalent gum not containing CPP-ACP in remineralization of enamel subsurface lesions in situ with mineral that is more resistant to subsequent acid challenge.

Ramalingam et al (2005)[58] studied the minimal concentration of casein phosphopeptide-stabilized amorphous calcium phosphate (CPP-ACP) which when added to a sports drink would eliminate such erosion in vitro using scanning electron microscopy (SEM).They concluded that adding casein phosphopeptide-stabilized amorphous calcium phosphate to the sports drink powerade and significantly reduced the beverage's erosivity without affecting the product's taste.

Manton et al (2006)[49] stated that inclusion of fluoride into CPP-ACP (as CPP-ACFP) forms a novel material with fluoride incorporated into the nanocomplex and also the advantages of increased remineralization are indicated for CPP-ACFP over CPP-ACP due to the availability of the fluoride ion with calcium and phosphate ions at the enamel surface, increasing fluorapatite formation. Clinically, CPP-ACP can be delivered to the tooth surface in several vehicles: chewing gum, lozenge, topical crème, mouthrinse, toothpaste and added to GIC restorative material.

Cochrane et al (2006)[13] stated that CPP-ACFP solutions remineralize enamel subsurface lesions *in vitro* by the deposition of fluorapatite increasing mineral content and improving the translucency.

Cross et al (2007)[15] stated that the CPP-ACP complexes readily incorporate fluoride ions forming casein phosphopeptide-amorphous calcium fluoride phosphate (CPP-ACFP). A mechanism is discussed which provides a rationale for the ability of the CPP-ACP to remineralize carious lesions in dental enamel. It is concluded that the CPP are a safe and novel carrier for calcium, phosphate and hydroxide (fluoride) ions to promote enamel remineralization with application in oral care products, dental professional products and foodstuffs.

Oshiro et al (2007)[55] evaluated the effect of CPP-ACP paste on demineralization by observing the treated tooth surface using FE-SEM. The SEM observations revealed different morphological features brought about by the various storage conditions.

Demineralization of the enamel and dentin surfaces was more pronounced with the control specimens. On the other hand, enamel and dentin specimens treated with CPP-ACP paste revealed slight changes in their morphological features.

Reynolds et al (2008)[61] determined the ability of CPP-ACP to increase the incorporation of fluoride into plaque and to promote enamel remineralization in situ.The addition of 2% CPP-ACP to the 450-ppm-F mouthrinse significantly increased the incorporation of fluoride into plaque.The dentifrice containing 2% CPP-ACP produced a level of remineralization similar to that achieved with a dentifrice containing 2800 ppm F and also the dentifrice containing 2% CPP-ACP plus 1100 ppm F was superior to all other formulations.

Tantbirojn et al (2008)[73] done an invitro study by using surface microhardness to evaluate whether a paste containing casein phosphopeptide amorphous calcium phosphate (CPP-ACP) can reharden tooth enamel softened by a cola drink, and how different saliva-substitute solutions affect the enamel hardness. They concluded that the application of CPP-ACP paste with continuous replenishment of saliva-like solution for 48 h significantly hardened enamel softened by a cola drink.

Kumar et al (2008)[43] investigated the efficacy of CPP-ACP containing tooth mousse on the remineralization of enamel lesions and compared its efficacy to that of a fluoride containing toothpaste using polarized light microscopy. They concluded that CPP-ACP containing Tooth Mousse remineralize initial enamel lesions and it showed a higher remineralizing potential when applied as a topical coating after the use of fluoridated toothpaste.

Neuhaus et al (2009)[54] stated that dental products with casein phosphopeptide--amorphous calcium phosphate-nanocomplexes (CPP-ACP) are used in several tooth products (toothpastes, chewing gums, mouthrinses) and are as well used in dental filling material. CPP-ACP containing products are supposed to enhance remineralisation of dental hard tissues and thus might play a major role in prevention and therapy of initial caries or erosively dissolved enamel.

Panich et al (2009)[56] conducted an in vitro study to compare the hardness of normal enamel with enamel eroded by a cola soft drink and enamel remineralized by casein phosphopeptide-amorphous calcium phosphate (CPP-ACP) or artificial saliva. They found that CPP-ACP increased the hardness of eroded enamel and also had a greater effect on enamel hardness than did artificial saliva and consumption of a cola soft drink can cause tooth erosion. CPP-ACP may significantly remineralize eroded enamel compared with artificial saliva.

Willershausen et al (2009)[78] demonstrated that there is a slight gain in the mineral contents after the application of a CPP-ACP paste, mainly in the upper enamel layer. The application of CPP-ACP paste may enhance the remineralisation after an erosive challenge and thus offer some protection for patients who are at risk for erosion.67

Sherine Badr et al (2010)[71] assessed the effect of acidulated phosphate fluoride gel (APF), sodium fluoride varnish (NaF) and casein phosphopeptide-amorphous calcium phosphate fluoride paste (CPP-ACPF) on the dental erosion produced by coca cola in primary and permanent teeth. They concluded that all of the tested fluoride treatments were able to reduce erosive enamel loss in both primary and permanent teeth. Primary and permanent enamel substrates reacted differently to different fluoridated compounds. CPP-ACPF paste is a promising remineralizing material.

N.Srinivasan, M.Kavitha, S.C.Loganathan (2010)[72] compared the remineralization potential of CPP–ACP and CPP–ACP with 900 ppm fluoride on eroded human enamel using Vickers surface microhardness measurements and scanning electron microscope analysis(SEM). They found that both CPP–ACP and CPP–ACP with 900 ppm fluoride substantially remineralized the softened enamel, with the CPP–ACP and fluoride combination showing higher remineralization potential than CPP–ACP.

Hamba et al (2011)[25] evaluated the effects of casein phosphopeptide-amorphous calcium phosphate (CPP-ACP) and CPP-ACP with 900 ppm fluoride (CPP-ACPF) pastes on inhibition of enamel demineralization over time, using polychromatic micro-computed tomography (micro-CT). The application of CPP-ACP or CPP-ACPF pastes to sound enamel surfaces resulted in inhibition of enamel demineralization and a better effect was noted for the latter paste.

Functionalised Tricalcium phosphate (fTCP):

In early 2009, 3M ESPE introduced two dentifrice formulations, one with 5,000 ppm fluoride (Clinpro™ 5000 Anti-Cavity Toothpaste) and one with 950 ppm fluoride (Clinpro™ Tooth Crème), that contain a calcium phosphate ingredient called TCP. TCP, or functionalized TCP, is a unique technology involving mechanochemical ball-milling of tri-calcium phosphate with simple organic ingredients that results in a functionalized or bioactive tri-calcium phosphate.

Karlinsey et al (2008)[32] studied the remineralization of white-spot enamel lesions from five treatment groups with deionized water, a paste with casein phosphopeptide-amorphous calcium phosphate (CPP-ACP) and 900 ppm fluoride, a 1,450 ppm fluoride dentifrice with calcium phosphate nano particles and protein,1,000 ppm fluoride unmodified dentifrice and a 1,000 ppm fluoride modified dentifrice with functionalized β-TCP (fTCP). The 1,000 ppm fluoride dentifrice with fTCP showed mean Vickers hardness recoveries and fluoride uptakes higher than those of the paste with CPP-ACP and 900 ppm fluoride and the unmodified 1,000 ppm fluoride dentifrice incorporating fTCP into a 1,000 ppm fluoride dentifrice may boost anti-caries performance.

Karlinsey et al (2008)[33] determined the ability of a conventional 5,000 ppm F dentifrice, a 5,000 ppm F dentifrice with Microdent® and a 5,000 ppm F dentifrice containing TCP. They concluded that the 5,000 ppm F dentifrice containing TCP exhibited greater rehardening than the unmodified 5,000 ppm F dentifrice with Microdent®.

Karlinsey et al (**2009)**[35] investigated the anti-erosion effects of 225 ppm fluoride plus an innovative form of tricalcium phosphate relative to 225 ppm fluoride using a pH cycling model comprising treatment saliva and acid challenge periods. Results indicate that synergistic effect can be produced when TCP is combined with fluoride and administered to eroded enamel.

Karlinsey et al (2009)[34] determined the fluoride dose response of experimental NaF dentifrices containing TCP (900 ppm F + CPP-ACP 5,000 ppm F, liquid gel 500 ppm F + TCP,950 ppm F + TCP,1,100 ppm F,5,000 ppm F, 5000 pm F + TCP) and evaluated relative enamel and dentin abrasivity. Results show that all dentifrice formulations exhibited acceptable enamel and dentin abrasivity although the dentifrice formulations with TCP and the formulation with CPP-ACP exhibited slightly lower dentin abrasivity than the 5,000 ppm fluoride dentifrice, liquid gel.

Pfarrer et al (**2009)**[57] acknowledged that fluoride remains the most widely used and thoroughly studied drug for remineralization purposes. However, they suggest that some calcium-containing technologies, including functionalized TCP, may be incorporated into topically applied fluoride-containing preparations without negatively affecting the proven benefits of fluoride.

Karlinsey et al (2009)[36] investigated the remineralization of white-spot enamel lesions with placebo, a conventional 1,100 parts per million (ppm) fluoride dentifrice, a paste with casein phosphopeptide-amorphous calcium phosphate (CPP-ACP) and

900 ppm fluoride, a 1,100 ppm fluoride dentifrice with ACP, a 950 ppm fluoride dentifrice with functionalized β-TCP (fTCP), a 5,000 ppm fluoride dentifrice with fTCP and a conventional 5,000 ppm fluoride dentifrice. The placebo resulted in the deepest indentations, while the two dentifrices containing fTCP and the 1,100 ppm fluoride dentifrice containing ACP resulted in the shallowest indentations. Therefore, sodium fluoride (NaF) formulations containing calcium technologies such as fTCP show promise in remineralizing white-spot lesions.

Mackey et al (2009)[48] evaluated the in vitro occlusion of dentin tubules from fluoride- and fluoride-free hypersensitivity dentifrices, including two prototype sodium fluoride (NaF) dentifrices containing a functionalized TCP (fTCP) technology, using a remineralization/demineralization model. Calcium sodium phosphosilicate dentifrice and paste with CPP-ACP and 900 ppm fluoride showed considerable occlusion and distilled water and conventional 1,100 ppm fluoride dentifrice showed virtually no occlusion. The dentifrice containing the fTCP technology showed complete tubule occlusion with mineral layer formation.

Karlinsey et al (**2010)**[37] discussed the development of a unique functionalized β-TCP (fTCP) using a solid-state mechanochemical process and evaluates its fluoride compatibility and remineralization efficacy. They concluded that fTCP intertwines synergistically with fluoride and the enamel tissue to produce superior remineralization of the subsurface lesion compared with fluoride alone.

Karlinsey et al (2010)[38] evaluated the efficacy of TCP-sodium lauryl sulfate (SLS) plus 5,000 parts per million (ppm) fluorides relative to 5,000 ppm fluoride alone in remineralizing weakened enamel emulating early caries formation with distilled water. TCP-SLS plus 5000 ppm fluoride significantly boosted remineralization of subsurface enamel lesions, with microhardness values increasing up to 30 percent greater than fluoride alone.

Amaechi et al (2010)[5] evaluated the remineralization potential of 225 parts per million fluoride (NaF) rinses with and without functionalized β-TCP on eroded enamel in a double-blind crossover in situ model and 225 ppm fluoride rinse with and without TCP The treatments were administered twice a day following one-minute brushing with a fluoride-free paste.All three treatments led to remineralization; however, the fluoride plus TCP rinse led to significantly more remineralization relative to saliva and the fluoride-only rinse.

Karlinsey et al (2010)[40] modified β-TCP with fumaric acid (FA) to create a promising surface and subsurface enamel mineralization system.TCP-FA material was prepared using mechanochemical ball milling. TCP-FA promoted enhanced remineralization compared with distilled water and mTCP, likely due to the functionalization of β-TCP and FA that occurred during milling.

Featherstone et al (2010)[22] established pH-cycling caries-simulation model to assess whether 5,000 parts per million (ppm) sodium fluoride (F) dentifrices with and without a functionalized β-TCP (fTCP) additive inhibit demineralization and/or promote remineralization. They found that the dentifrice containing fTCP did not interfere with fluoride, performed at least as well as the clinically proven 5,000 ppm fluoride dentifrice and appeared to exhibit the greatest protection against lesion progression.

Hogan et al (2010)[28] evaluated the demineralization and remineralization effects on enamel lesions after treatment with dentifrices containing fluoride and calcium phosphate (CaP) using a pH-cycling model in vitro with conventional 1,100 ppm fluoride dentifrice diluted to a 110 parts per million (ppm) fluoride control (CCPD), a paste with CPP-ACP and 900 ppm fluoride (MIPP), a 950 ppm fluoride dentifrice (CPTC), conventional 1,100 ppm fluoride dentifrice (CCPP), a 5,000 ppm fluoride dentifrice with TCP (CP5K) and a conventional 5,000 ppm fluoride dentifrice (PREV). After cycling, the low-fluoride group showed the most lesion progression, while the 5,000 ppm fluoride dentifrice with TCP and the conventional 5,000 ppm fluoride dentrifice showed the least progression and were not significantly different from each other.

Karlinsey et al (2010)[39] determined the anti-caries potential of two dentifrices, one with fast dispersion for improved enamel fluoride uptake and one containing an innovative TCP system for enhanced remineralization, in an *in vitro* pH cycling model with fluoride-free dentrifrice, a 5,000 ppm fluoride dentifrice, liquid gel and a 5,000 ppm fluoride dentifrice with TCP. The groups cycled between four 1-minute treatment periods and one 4-hour acid challenge per day for 10 days, interspersed with immersion in artificial saliva.The result showed that the dentifrice containing fTCP imparted superior remineralization at both the enamel surface and within the subsurface lesion and may provide more significant anti-caries benefits than fluoride-only and fluoride-free dentifrices.

Karlinsey et al (2011)[41] evaluated the *in vitro* remineralization effects of four dentifrice systems1) placebo (0 ppm F), 2) 500 ppm F, 3) 1,150 ppm F and 4) 500 ppm F plus functionalized tri-calcium phosphate (fTCP) using microhardness and fluoride uptake analysis.Results revealed significant differences among the four groups, with the placebo and 500 ppm F dentifrices providing significantly less remineralization relative to the 1,150 ppm F and 500 ppm F plus fTCP dentifrices.

Rirattanapong et al (2011)[65] assessed the effect of five different dental products on surface microhardness of enamel exposed to chlorinated water in vitro with artificial saliva, 1,000 ppm fluoride toothpaste, CPP-ACP paste, CPP-ACP with 900 ppm fluoride paste, CPP toothpaste and tricalcium phosphate with 950 ppm fluoride paste. After remineralization, the mean surface microhardness of the artificial saliva group was significantly less than the other groups. Five different dental products (1,000 ppm fluoride toothpaste, CPP-ACP paste, CPP-ACP with 900 ppm fluoride paste, CPP toothpaste and tricalcium phosphate with 950 ppm fluoride paste) increased the hardness in vitro of eroded enamel caused by chlorinated water.

Amaechi et al (2012)[6] evaluated the ability of a high-fluoride dentifrice containing tricalcium phosphate to remineralize white spot lesions and inhibit lesion formation. The results indicate that combining fluoride with tricalcium phosphate could provide more anti-caries benefits compared to using fluoride alone.

Rirattanapong et al (2012)[66] compared the remineralization potential of dental products containing calcium on human enamel softened by soft drinks with artificial saliva, 1,000 ppm fluoride toothpaste, CPP-ACP paste, CPP-ACP with 900 ppm fluoride paste and tricalcium phosphate with 950 ppm fluoride paste.The CPP-ACP paste, CPP-ACP with 900 ppm fluoride paste and tricalcium phosphate with 950 ppm fluoride paste treatments all increased the hardness of the teeth in vitro.

Mathews et al (2012)[50] evaluated the remineralization of eroded enamel by NaF rinses in an intra-oral model using fluoride-free (0 ppm F), 225 ppm F, 225 ppm F plus functionalised β-tricalcium phosphate (fTCP), and 450 ppm. They demonstrated that addition of fTCP to an aqueous rinse containing 225 ppm F may provide significant remineralisation benefits. Therefore, the combination of relatively low levels of fluoride and fTCP might be an effective alternative to a high fluoride treatment for anti-erosion benefits.

Karlinsey et al (2012)[42] stated that fTCP is a low-dose system designed to fit within existing topical fluoride preparations. The functionalization of β-TCP with organic and/or inorganic molecules provides a barrier that prevents premature fluoride-calcium interactions and aids in mineralization when applied *via* common preparations and procedures.

Mithra N Hegde et al (2012)[53] evaluated the effect of cola based beverage on the calcium loss of enamel surface pre-treated with fluoride enriched casein phosphopeptide amorphous calcium phosphate (CPP-ACPF) and beta-tricalcium phosphate (β-TCP) using energy dispersive X-ray analysis (EDX). They concluded that β-TCP was found to be more effective than CPP-ACPF in inhibiting the de-mineralization caused by cola based beverage.

MATERIALS AND METHODS:

The following armamentaria and materials were used in this study:

Armamentarium:

For mounting of enamel slabs:

1. 15 unerupted (i.e. not exposed to oral environment), matured human third molar teeth.
2. 10% formalin
3. Diamond disc
4. Micromotor straight handpiece (NSK,JAPAN)
5. Standardized mould for acrylic resin block. (1 cm diameter,1 cm height cylinder)
6. Autopolymerizing resin (DPI)
7. Porcelain cup and spatula

For polishing of enamel slabs:

8. Grit silicon carbide paper (240,400, 600 size)
9. Metallographic polisher (Magnum opus international,India)

For storing of enamel slabs:

8. Artificial Saliva:

Compositions of artificial saliva-(McKnight-Hanes,Whitford)-1992

1. Methyl –p-hydroxybenzoate -2.00 **(grams per liter)**
2. Sodium Carboxy methyl Cellulose - 10.00
3. KCl - 0.625
4. $Mgcl_2$.6H20 - 0.059
5. K_2HPO_4 -0.166
6. KH_2PO_4 -0.326
7. Distilled water to make volume of 1 liter.

The pH of artificial saliva was adjusted to 6.75 with KOH.

For Testing of enamel slabs:

9. Vickers Microhardness tester (Bareiss, Digi test, V test, Germany).
10. Scanning Electron Microscope (SEM) (Hitachi 3400 N, Japan) and Gold ion sputtering machine (Hitachi E 1010 Ion sputter).

EXPERIMENTAL MATERIALS USED:

NAME	**MANUFACTURER**	**COMPOSITION**
GC Tooth Mousse Plus	GC Corporation Tokyo Japan.	Sodiumfluoride-0.2%w/w(900ppm), Glycerol -20.0% Propylene glycol- 2.0% CPP-ACP (casein phosphopeptide - amorphous calcium Phosphate) -10.0% D-glucitol- 5% Colloidal silica -2.0%

		Sodium carboxyl methyl cellulose(CMC-Na)- 2% Titanium dioxide- 2% Xylitol- 2% Guar gum <0.1% Phosphoric acid <0.2% Sodium saccharin <0.2% Zinc oxide <0.1% Magnesium oxide <0.1% Ethyl 4-hydroxybenzoate <0.1%, Propyl4-hydroxybenzoate 0.1%, Fluoride-900ppm.
Clinpro Tooth Creme	3M ESPE ST.Paul,USA	Water,sorbitol,hydrated silica, glycerin, polyethlene-polypropylene glycol,flavour, polyethylene glycol, sodium lauryl sulphate, titanium dioxide, carboxymethyl cellulose, sodium saccharin,sodium fluoride (950ppmF,0.21%w/w,0.12%w/v fluoride ion),tricalcium phosphate.
Coca cola	Classic coke, India	Carbonated water, sugar (which can be sucrose or high-fructose corn syrup), caffeine, phosphoric acid, caramel, natural flavorings (which include coca leaf extract).

METHODOLOGY:

COLLECTION OF TEETH

15 unerupted (i.e. not exposed to oral environment) matured, human third molar teeth without any defects (cracks) were collected for this study and stored in 10% formalin till enamel slab preparation.

ENAMEL SLAB PREPARATION:

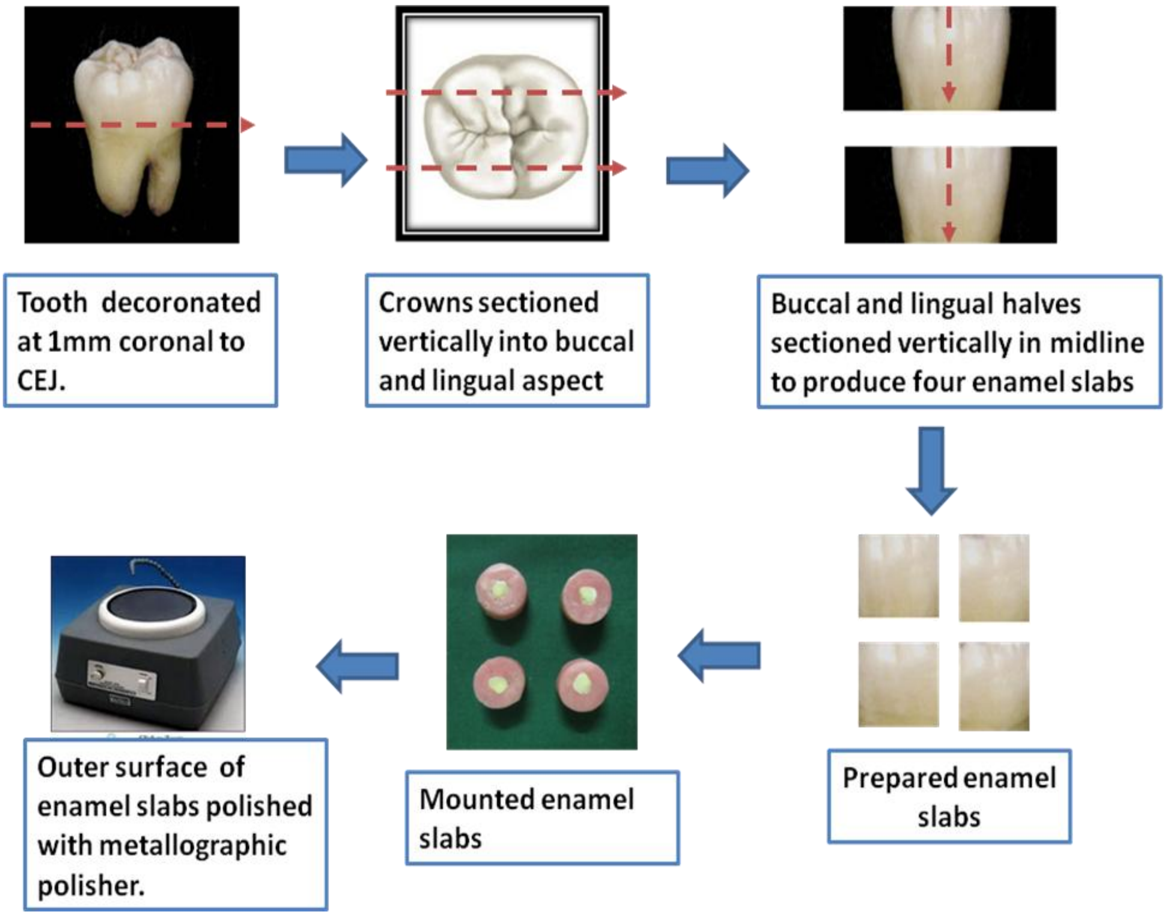
All teeth were rinsed under tap water in order to remove blood and tissue debris followed by removal of radicular part by decoronating the teeth 1mm coronal to CEJ using water-cooled diamond disk.

Crowns were sectioned vertically into buccal and lingual aspects followed by sectioning of buccal half into 2 equal halves and lingual half into 2 equal halves. So each tooth provides 4 enamel slabs and totally 60 enamel slabs with approximate dimensions of 4mmx4mmx2mm and had been stored in 10% of formalin (pH 7.0) at room temperature (25ºC).

MOUNTING AND POLISHING OF THE ENAMEL SLABS:

Enamel slabs were mounted on an acrylic resin block using standardized mould and their enamel surfaces were polished with 240,400, 600 size grit silicon carbide paper to obtain flat and smooth surfaces followed by polishing with metallographic polisher at low speed. This polishing procedure removed approximately 200 µm of the tooth surface.

ENAMEL SLAB PREPARATION:



PHASE I:

a) SURFACE MICROHARDNESS MEASUREMENT AND SEM (SCANNING ELECTRON MICROSCOPE) ANALYSIS.

(i) VICKERS MICROHARDNESS TESTING:

Vickers hardness number (VHN) was determined by making three indentations in different regions of each specimen using a square based diamond pyramid Vicker's indenter under a load of 100 g for 10 s. The indentations were made 100 µm apart from each other to avoid residual stress. This procedure resulted in well defined indentations.The main criteria for accepting an indentation were clearness of outline and absence of flaws in tooth in the area of measurement.Microhardness testing were carried out for all enamel slabs and results were obtained.In order to standardize the enamel slabs, 45 slabs with VHN in a range of 330.0 to 349.6 were selected for the study.

(ii) SEM EVALUATION:

Enamel slabs were mounted on metal base followed by sputtering with gold in sputtering machine.The coating is done in order to enhance the number of secondary electrons from the surface of the enamel slabs.

Then the enamel slabs were examined under a Scanning Electron Microscope and viewed at 500 x and 1000 x.

(b) Enamel softening:

After the baseline microhardness and SEM analysis, gold sputtering in enamel slabs were removed and followed by immersing in 6ml of cola drink for 8 minutes at room temperature.The cola drink was replenished every 2 min.

After demineralization with cola drink the softened enamel slabs were again taken for surface microhardness measurement and SEM analysis.

DISTRIBUTION OF THE ENAMEL SLABS:

Enamel Slabs were randomly divided into three groups:

Group I: **Control** (n=15)

Group II: **CPP-ACFP** (n=15)

Group III: **functionalised Tricalcium Phosphate (fTCP)** (n=15)

PHASE II:

The softened enamel slabs were subjected to three different remineralization protocols.

GROUP I: 15 enamel slabs were stored in **artificial saliva.**

GROUP II: 15 enamel slabs were subjected to **CPP-ACFP** paste application with cotton tip applicator for three minutes at **0, 8, 24, 36 hours** followed by washing in distilled water and storing in artificial saliva.

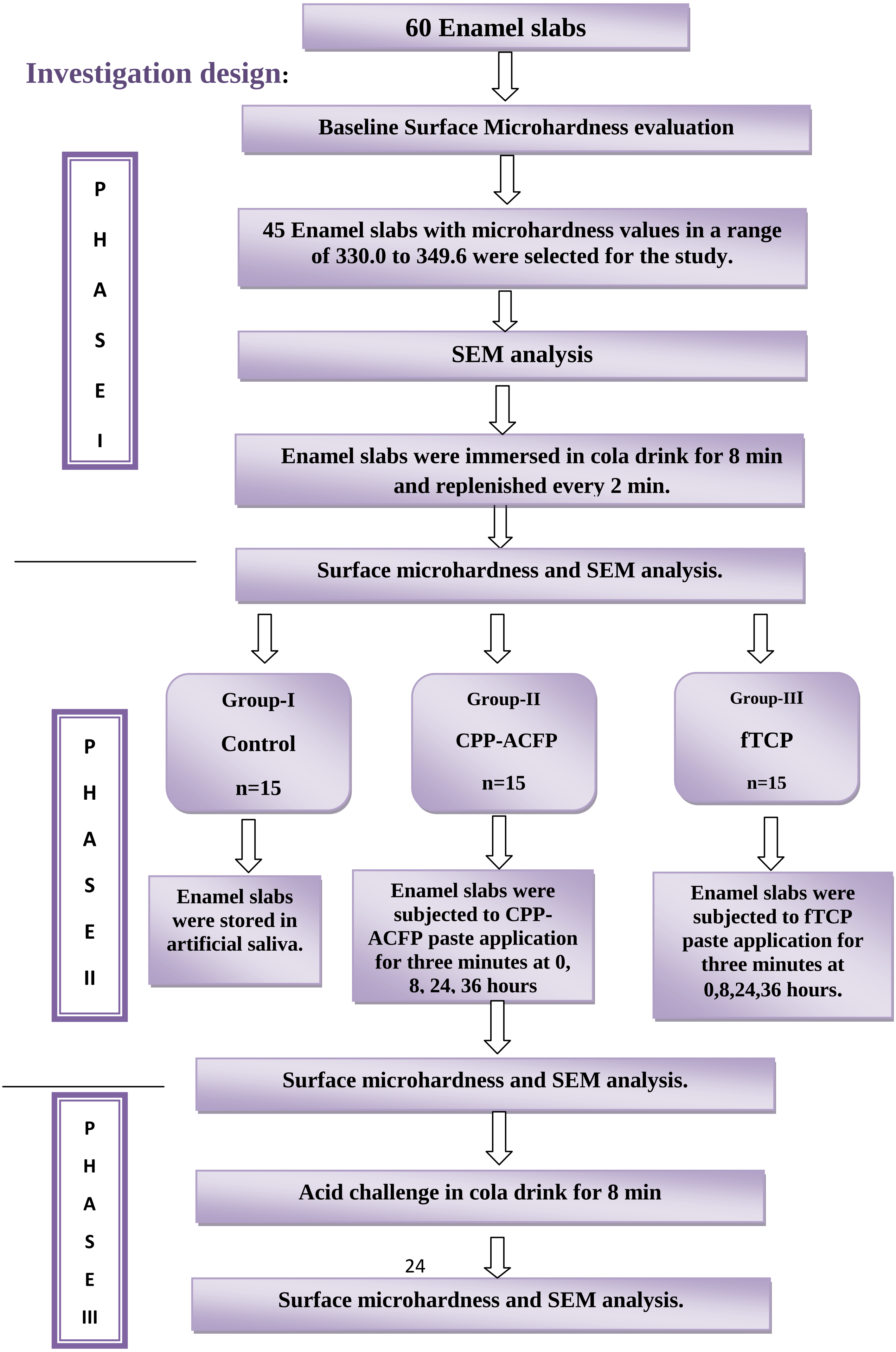
GROUP III: 15 enamel slabs were subjected to **fTCP** paste application with cotton tip applicator for three minutes at **0, 8, 24, 36 hours** followed by washing in distilled water and storing in artificial saliva.

After remineralization protocols, enamel slabs were taken for assessment of surface microhardness changes and SEM evaluation.

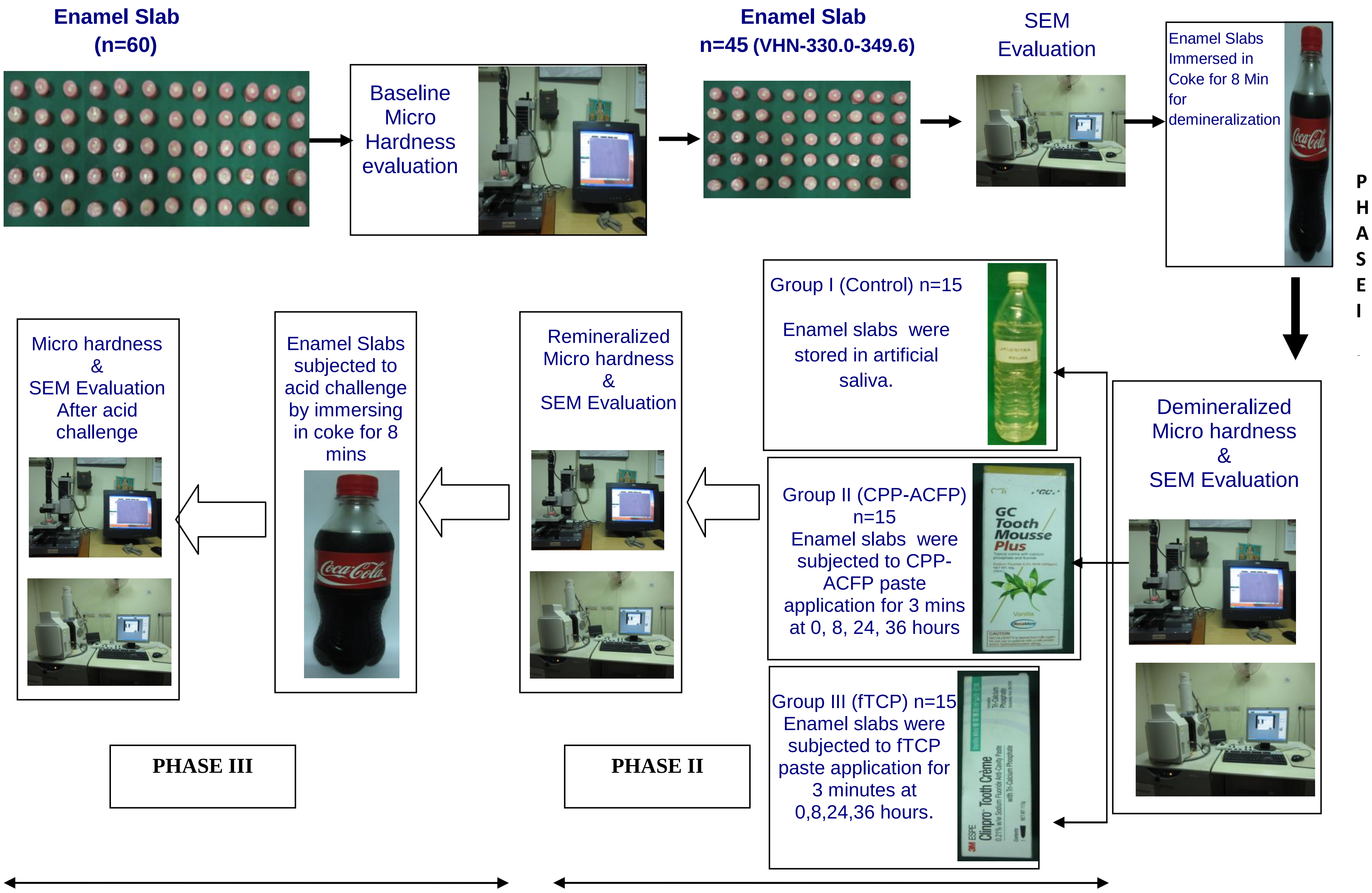
PHASE III:

After analyzing the surface microhardness and SEM evaluation, gold sputtering in enamel slabs were removed and followed by immersing in 6ml of cola drink for 8 minutes at room temperature for acid challenge .The cola drink was replenished every 2min. Then the enamel slabs were washed with distilled water and again taken for final assessment of surface microhardness changes and SEM evaluation.

Investigation design:



STUDY DESIGN



ARMAMENTARIUM

FIG 1: UNERUPTED THIRD MOLARS

Fig 2: INSTRUMENTS, MATERIALS USED

FIG 3: MOUNTED ENAMEL SLABS

FIG 4: METALLOGRAPHIC POLISHER

Fig 5: ENAMEL SLABS DISTRIBUTION

Fig.6: COLA DRINK (CLASSIC COKE INDIA)

Fig 7: ARTIFICIAL SALIVA

EXPERIMENTAL MATERIALS USED:

Fig.8: CPP-ACFP PASTE (GC TOOTH MOUSSE PLUS, GC CORPORATION)

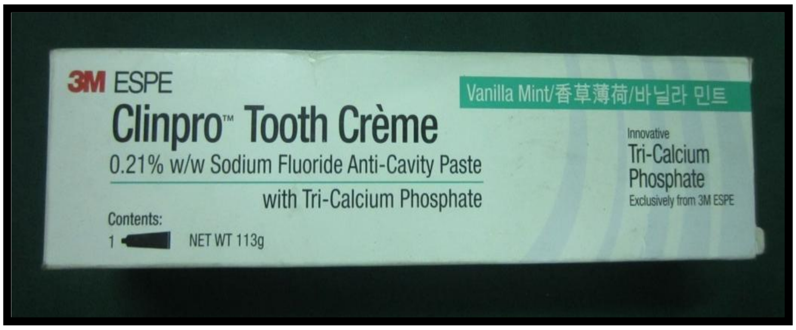


Fig 9: fTCP PASTE (CLINPRO, 3M ESPE)

Fig 10: ENAMEL SLABS IMMERSED IN COLA DRINK

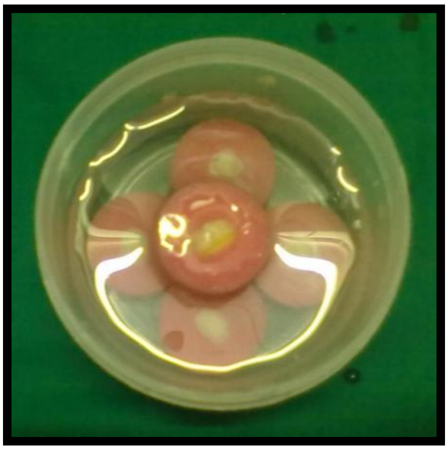


Fig 11: ENAMEL SLABS STORED IN ARTIFICIAL SALIVA

Fig 12: CPP-ACFP PASTE APPLICATION ON GROUP II ENAMEL SLABS

Fig 13: fTCP PASTE APPLICATION ON GROUP III ENAMEL SLABS

Fig 14: VICKERS MICROHARDNESS TESTER (BAREISS,DIGI TEST,GERMANY)

FIG 15: GOLD ION SPUTTERING MACHINE (HITACHI E 1010 ION SPUTTER).

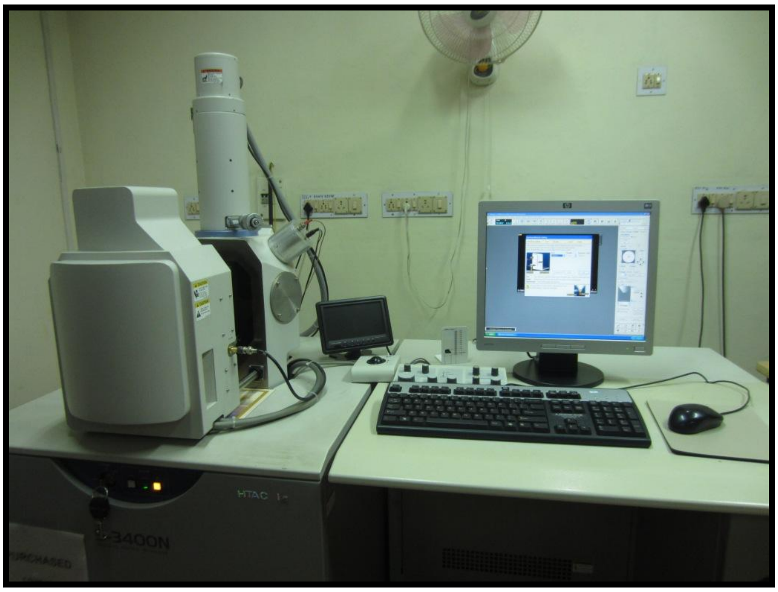


FIG 16: SCANNING ELECTRON MICROSCOPE (SEM) (HITACHI 3400 N, JAPAN)

Fig 17: ENAMEL SLABS POSITIONING IN SEM

RESULTS

TABLE 1: BASELINE SURFACE MICROHARDNESS VALUES:

Enamel Slab no.	Microhardness values (VHN)
1	348.6
2	335.4
3	332.7
4	310.4
5	337.3
6	342.7
7	346.4
8	315.5
9	339.5
10	349.6
11	340.5
12	283.5
13	330.8
14	336.6
15	344.1
16	298.5
17	342.7
18	341.1
19	331.8
20	320.6
21	339.5
22	342.8
23	331.4
24	361.4
25	349.6
26	338.5
27	340.5
28	354.7
29	330.1
30	334.6

Enamel Slab no.	Microhardness values (VHN)
31	336.3
32	358.7
33	341.1
34	339.9
35	342.5
36	288.4
37	344.7
38	346.2
39	339.5
40	294.4
41	348.4
42	335.8
43	346.2
44	305.5
45	331.8
46	342.5
47	337.3
48	311.1
49	336.6
50	344.1
51	332.7
52	324.6
53	349.6
54	340.5
55	337.3
56	309.7
57	339.5
58	341.1
59	348.6
60	358.7

Table shows the microhardness values of 60 enamel slabs ranging from 283.5 to 361.4 VHN

TABLE 2: 45 Enamel slabs with Microhardness values of 340±10 VHN:

Enamel slab no.	Baseline microhardness value (VHN)	Demineralized microhardness value (VHN)
1	348.6	264.6
2	335.4	255.8
3	332.7	260.2
4	337.3	259.2
5	342.7	261.3
6	346.4	270.1
7	339.5	258.5
8	349.6	266.7
9	340.5	262.3
10	330.8	259.8
11	336.6	254.3
12	344.1	264.1
13	342.7	268.7
14	341.1	262.3
15	331.8	258.5
16	339.5	265.4
17	342.8	270.1
18	331.4	264.3
19	349.6	261.3
20	338.5	257.4
21	340.5	266.3
22	330.1	254.3
23	334.6	269.7
24	336.3	252.8
25	341.1	260.3
26	339.9	270.4
27	342.5	264.6
28	344.7	262.5
29	346.2	274.3
30	339.5	261.6
31	348.4	262.3
32	335.8	258.8
33	346.2	266.4
34	331.8	254.3
35	342.5	267.6
36	337.3	259.8
37	336.6	255.8
38	344.1	261.3
39	332.7	264.1
40	349.6	274.3
41	340.5	262.3
42	337.3	269.7
43	339.5	255.8
44	341.1	268.4
45	348.6	272.6

Table 3: Microhardness values for Group I (Control)

Enamel slabs no.	Baseline (VHN)	Demineralized (VHN)	Remineralized (VHN)	Acid Challenge (VHN)
1	348.6	264.6	282.2	236.3
2	335.4	255.8	273.9	229.7
3	332.7	260.2	280.3	233.1
4	337.3	259.2	278.7	234.6
5	342.7	261.3	281.5	231.8
6	346.4	270.1	288.6	241.3
7	339.5	258.5	275.7	226.9
8	349.6	266.7	288.3	240.1
9	340.5	262.3	283.6	239.5
10	330.8	259.8	279.2	236.7
11	336.6	254.3	274.4	229.4
12	344.1	264.1	283.7	238.5
13	342.7	268.7	278.1	237.2
14	341.1	262.3	281.4	233.6
15	331.8	258.5	274.9	232.3

Table 4: Microhardness values for Group II- (CPP-ACFP).

Enamel slabs no.	Baseline (VHN)	Demineralized (VHN)	Remineralized (VHN)	Acid Challenge (VHN)
1	339.5	265.4	305.1	270.4
2	342.8	270.1	302.4	268.3
3	331.4	264.3	308.6	272.9
4	349.6	261.3	298.7	262.3
5	338.5	257.4	294.2	264.2
6	340.5	266.3	301.4	269.7
7	330.1	254.3	297.6	266.8
8	334.6	269.7	311.4	268.6
9	336.3	252.8	291.6	258.4
10	341.1	260.3	296.4	263.2
11	339.9	270.4	311.4	274.7
12	342.5	264.6	304.7	266.3
13	344.7	262.5	298.8	265.4
14	346.2	274.3	311.4	270.4
15	339.5	261.6	302.4	266.3

Table 5: Microhardness values for Group III- (fTCP)

Enamel slabs no.	Baseline (VHN)	Demineralized (VHN)	Remineralized (VHN)	Acid Challenge (VHN)
1	348.4	262.3	320.8	296.4
2	335.8	258.8	318.6	295.6
3	346.2	266.4	323.2	299.3
4	331.8	254.3	313.4	289.7
5	342.5	267.6	326.3	304.5
6	337.3	259.8	318.6	292.4
7	336.6	255.8	314.5	289.3
8	344.1	261.3	320.7	297.6
9	332.7	264.1	317.4	290.2
10	349.6	274.3	331.6	307.4
11	340.5	262.3	320.7	293.6
12	337.3	269.7	326.3	306.4
13	339.5	255.8	315.6	287.6
14	341.1	268.4	328.9	321.7
15	348.6	272.6	331.3	308.2

Table 6: Mean microhardness values (VHN) of each group:

Groups	**Baseline**	**Demineralized**	**Remineralized**	**Acid Challenge**
Control	339.99	261.76	280.30	234.73
CPP-ACFP	339.81	263.68	302.46	267.19
f-TCP	340.80	263.56	321.86	298.66

STATISTICAL ANALYSIS:

Datas were statistically analyzed using ONE WAY ANOVA and TUKEY HSD POST HOC multiple comparisons at 0 .05 level significance.

Table 7: ANOVA- Percentage increase in Microhardness (Remineralized):

	Sum of Squares	df	Mean Square	F	Sig.
Between Groups	.170	2	.085	588.141	.000
Within Groups	.006	42	.000		
Total	.176	44			

Table 8: Percentage increase in Microhardness

Descriptives:

	N	Mean	Std. Deviation	Std. Error	95% Confidence Interval for Mean		Minimum	Maximum
					Lower Bound	Upper Bound		
Control	15	.0707	.01280	.00330	.0636	.0778	.03	.08
CPP-ACFP	15	.1470	.01334	.00344	.1396	.1544	.12	.17
fTCP	15	.2213	.00961	.00248	.2160	.2267	.20	.23
Total	45	.1463	.06331	.00944	.1273	.1653	.03	.23

Table 9: Multiple Comparisons: Percentage increase in Microhardness

Tukey HSD

(I) Group	(J) Group	Mean Difference (I-J)	Std. Error	Sig.	95% Confidence Interval	
					Lower Bound	Upper Bound
Control	CPP-ACFP	-.07629*	.00439	.000	-.0870	-.0656
	fTCP	-.15066*	.00439	.000	-.1613	-.1400
CPP-ACFP	Control	.07629*	.00439	.000	.0656	.0870
	fTCP	-.07437*	.00439	.000	-.0850	-.0637
fTCP	Control	.15066*	.00439	.000	.1400	.1613
	CPP-ACFP	.07437*	.00439	.000	.0637	.0850

*. **The mean difference is significant at the 0.05 level.**

Table 10: Percentage decrease in micro hardness (Acid Challenge)

ANOVA

	Sum of Squares	df	Mean Square	F	Sig.
Between Groups	.061	2	.031	209.027	.000
Within Groups	.006	42	.000		
Total	.068	44			

Table 11: Percentage decrease in micro hardness (Acid Challenge)

Descriptives

	N	Mean	Std. Deviation	Std. Error	95% Confidence Interval for Mean		Minimum	Maximum
					Lower Bound	Upper Bound		
Control	15	.1627	.00961	.00248	.1573	.1680	.15	.18
CPP-ACFP	15	.1163	.00992	.00256	.1108	.1218	.10	.14
fTCP	15	.0722	.01579	.00408	.0635	.0810	.02	.09
Total	45	.1171	.03917	.00584	.1053	.1288	.02	.18

Table 12: Multiple Comparisons: Percentage decrease in micro hardness (Acid Challenge)

Tukey HSD

(I) Group	(J) Group	Mean Difference (I-J)	Std. Error	Sig.	95% Confidence Interval	
					Lower Bound	Upper Bound
Control	CPP-ACFP	.04635*	.00442	.000	.0356	.0571
	fTCP	.09043*	.00442	.000	.0797	.1012
CPP-ACFP	Control	-.04635*	.00442	.000	-.0571	-.0356
	fTCP	.04408*	.00442	.000	.0333	.0548
fTCP	Control	-.09043*	.00442	.000	-.1012	-.0797
	CPP-ACFP	-.04408*	.00442	.000	-.0548	-.0333

*. **The mean difference is significant at the 0.05 level.**

Interpretation of results for microhardness values:

Analysis of mean values of microhardness at 0.05 level significance revealed that

1. Enamel slabs on immersing in cola drink for 8 minutes resulted in **23% reduction** in microhardness values (Table 6).

Percentage increase in microhardness values after remineralization:

2. Group I (**Control**) demineralized enamel slabs resulted in **7% increase** in microhardness values after immersing in artificial saliva for 36 hrs (Graph 2).
3. Group II (**CPP-ACPF**) demineralized enamel slabs resulted in **14.70 % increase** in microhardness values after CPP-ACFP paste application for 3 min at 0,8,24,36 hrs (Graph 2).
4. Group III (**fTCP**) demineralized enamel slabs resulted in **22.1% increase** in microhardness values after f-TCP paste application for 3 min at 0,8,24,36 hrs(Graph 2).
5. Group III (**fTCP**) > Group II (**CPP-ACPF**) >Group I (**Control**)
6. Groups II and III were statistically significant to each other ($p<0.05$).

   (Table: 7)

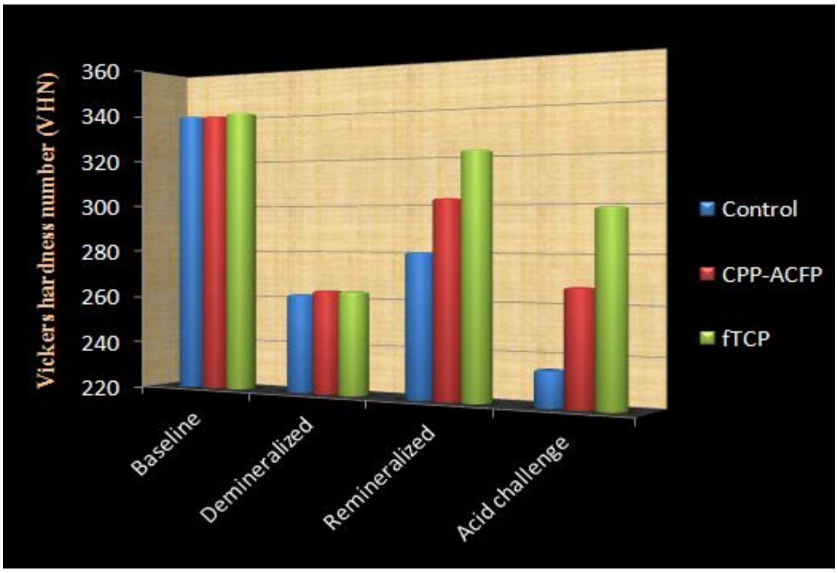
Percentage decrease in microhardness values after acid challenge:

7. Group I (**Control**) remineralized enamel slabs on acid challenge with coke for 8 minutes resulted in **16% reduction** in microhardness values (Graph 3).
8. Group II (**CPP-ACFP**) remineralized enamel slabs on acid challenge with coke for 8 minutes resulted in **11.60 % reduction** in microhardness values (Graph 3).
9. Group III (**fTCP**) remineralized enamel slabs on acid challenge with coke for 8 minutes resulted in **7.22 % reduction** in microhardness values (Graph 3).

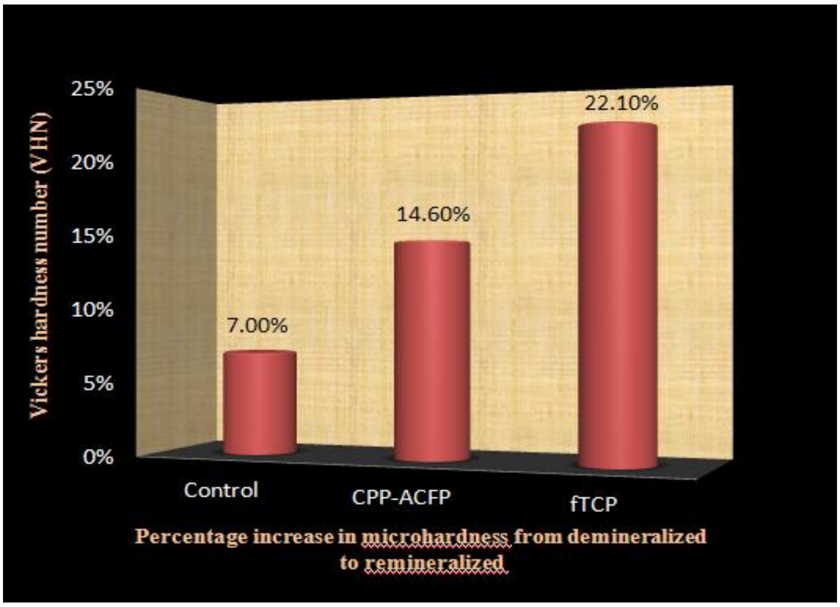
10. Group III (**fTCP**) < Group II (**CPP-ACFP**) < Group I (**Control**)

11. Groups II and III were statistically significant to each other ($p<0.05$) (Table: 10).

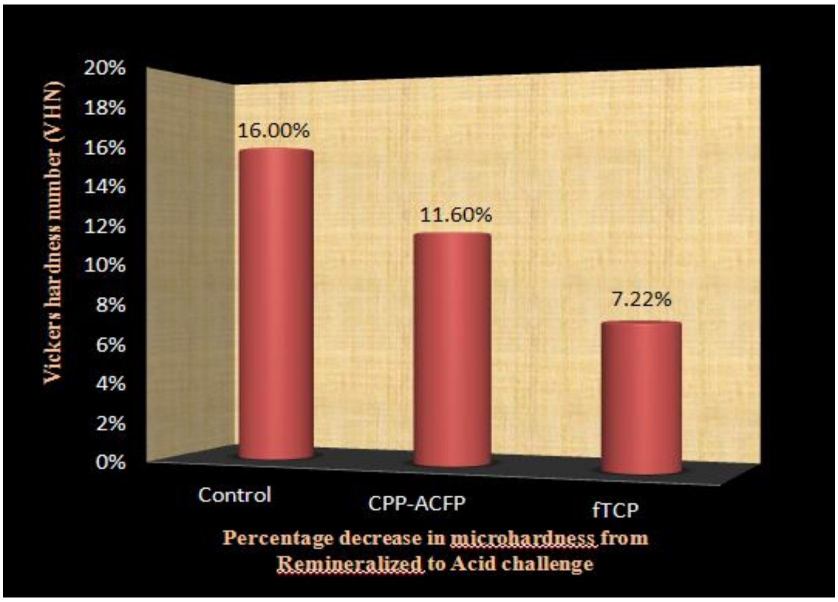
Graph 1: Comparison of Surface microhardness in different phases:



Graph 2: Comparison of percentage increase in surface microhardness after remineralization:

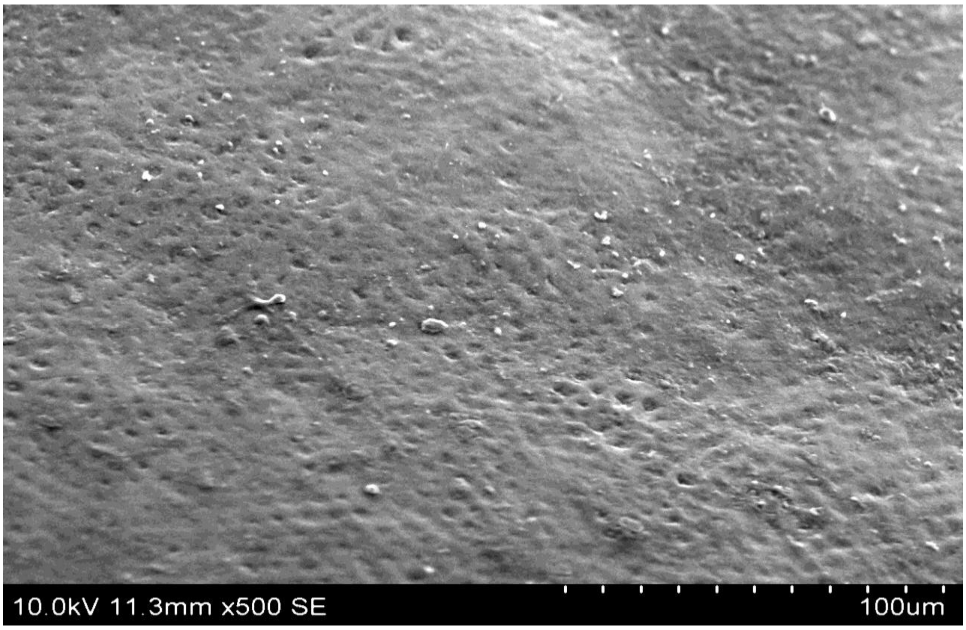


***Graph 3*: Comparison of percentage decrease in surface microhardness after acid challenge:**



SCANNING ELECTRON MICROSCOPIC (SEM) OBSERVATIONS

UNTREATED ENAMEL SURFACE:



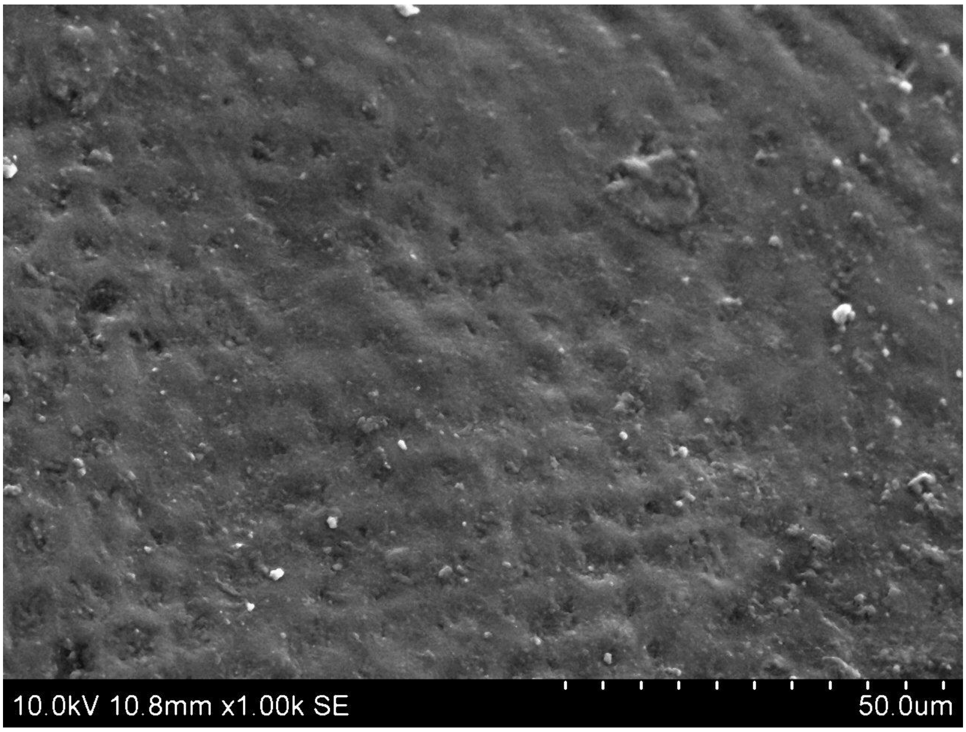
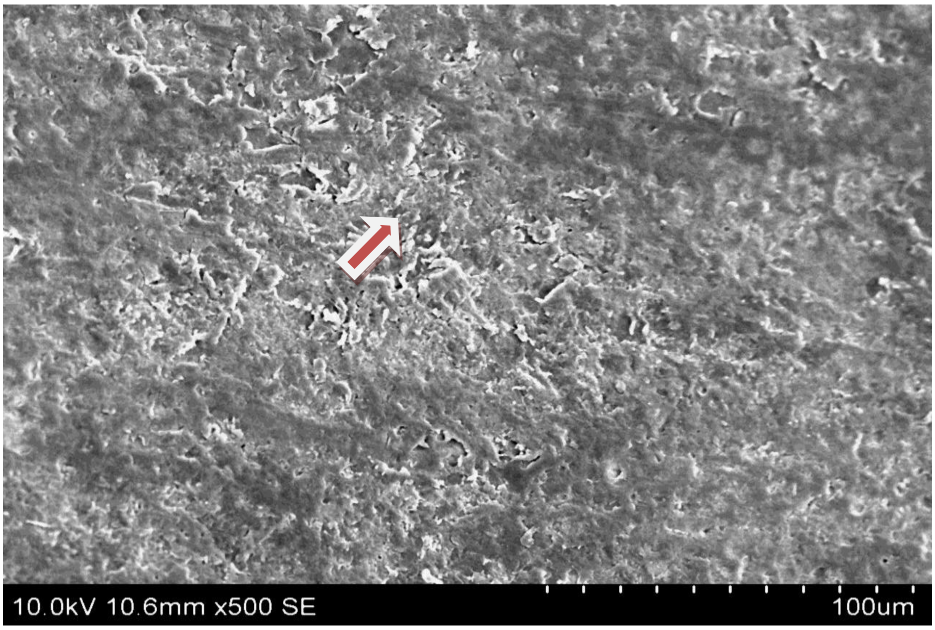


Fig 18: SEM images of intact enamel shows the relatively smooth surfaces before erosion attack when viewed at 500x, 1000x (original magnification).

DEMINERALIZED ENAMEL SURFACE:



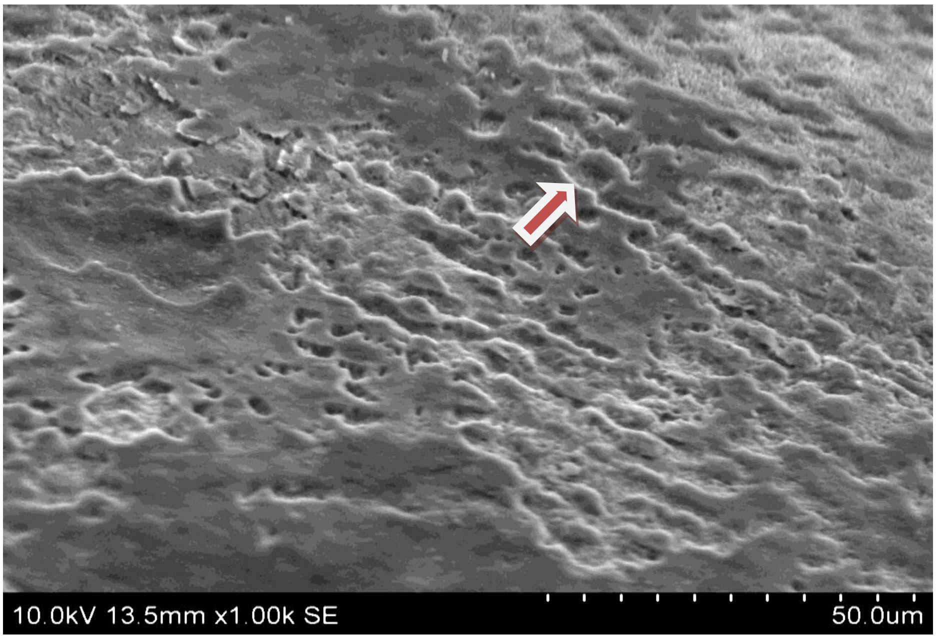


Fig 19: Enamel surfaces after immersing in cola drink for 8 min resulted in irregular surfaces with porosities indicating the demineralization.

REMINERALIZED ENAMEL SURFACE:

Group I (CONTROL):

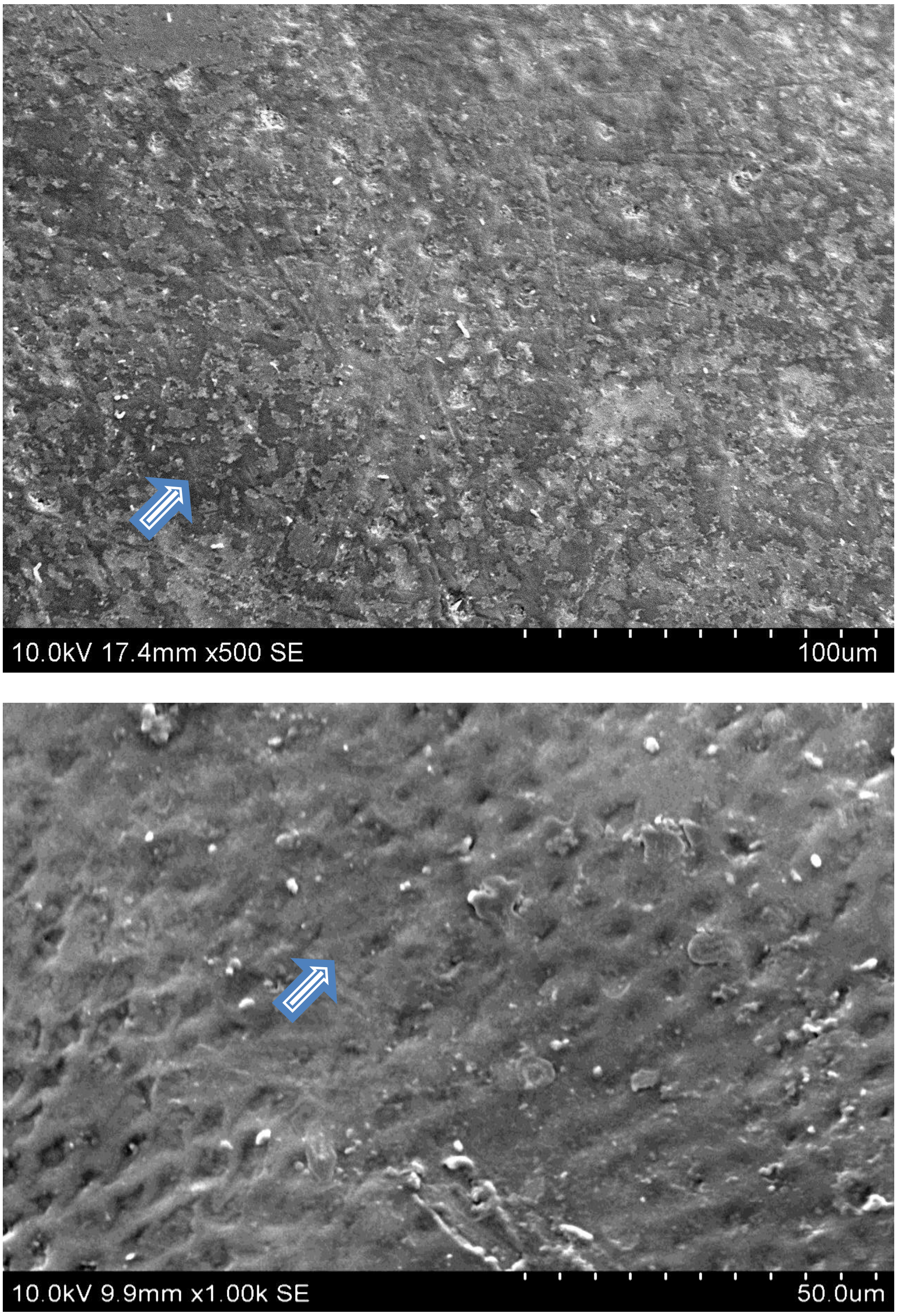
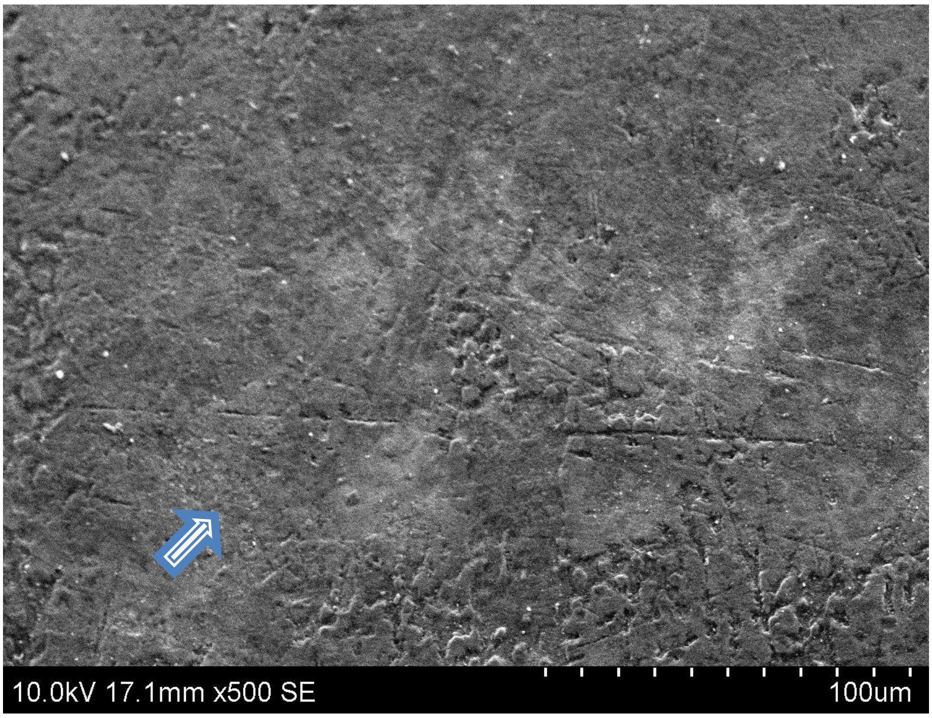


Fig 20: Enamel Surfaces after treatment with artificial saliva alone.Reduction in porosities visible but with inadequate remineralization.

REMINERALIZED ENAMEL SURFACE

Group II (CPP-ACFP):



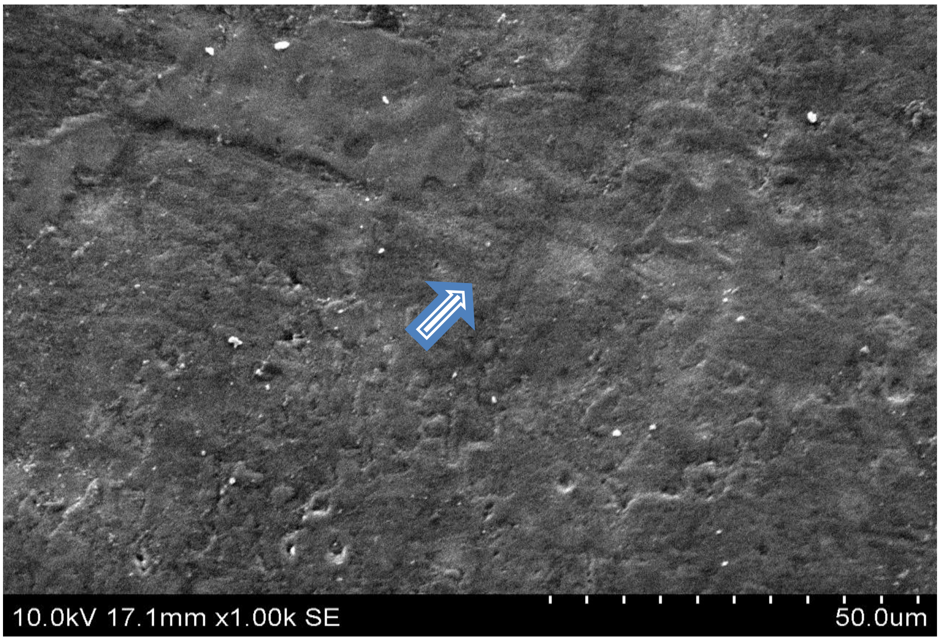
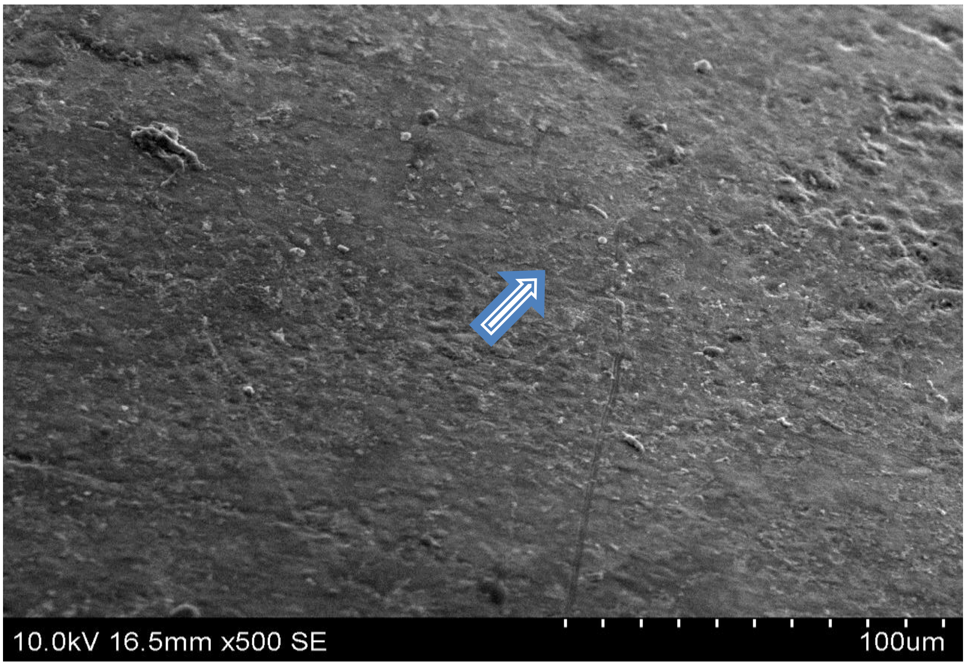


Fig 21: Enamel surfaces after treatment with CPP-ACFP results in adequate remineralization with reduction in porosities.

REMINERALIZED ENAMEL SURFACE

GROUP III (fTCP):



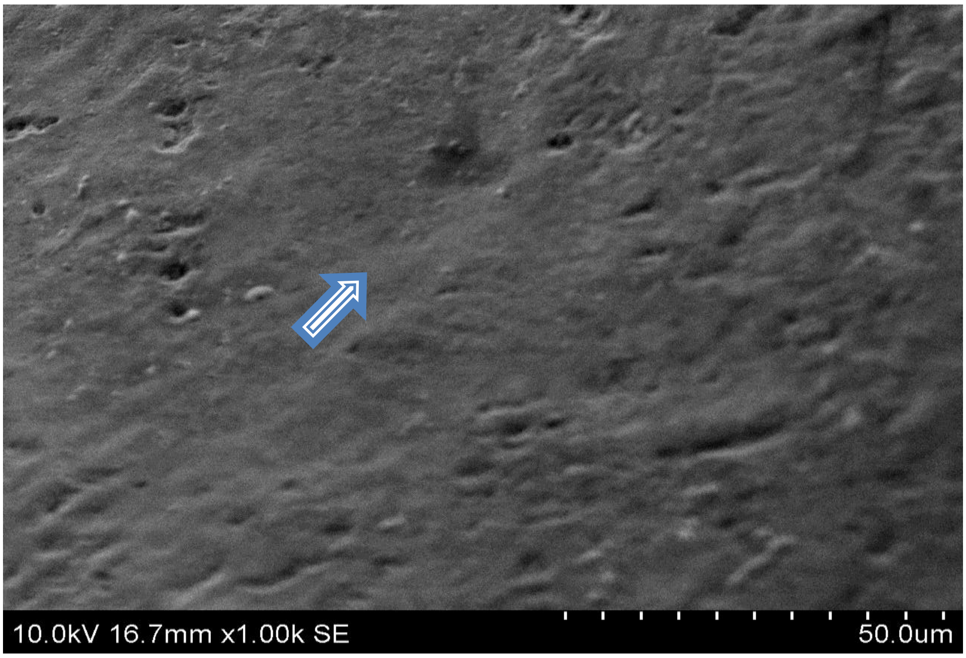
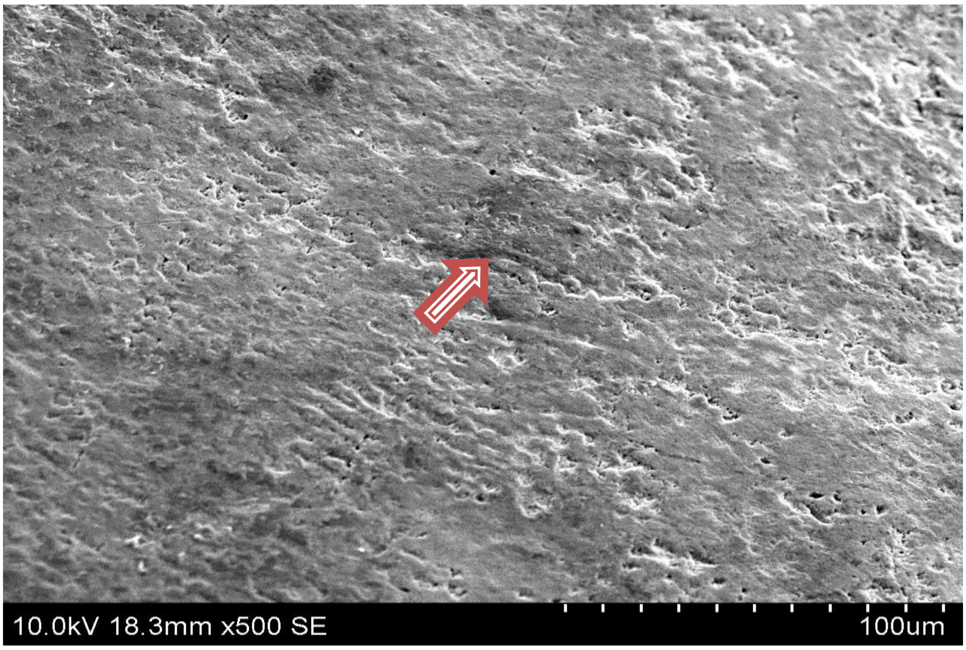


Fig 22: Enamel surfaces after treatment with fTCP results in enhanced remineralization throughout the surfaces with more homogenous morphology.

ACID CHALLENGE OF REMINERALIZED ENAMEL:

GROUP I (CONTROL):



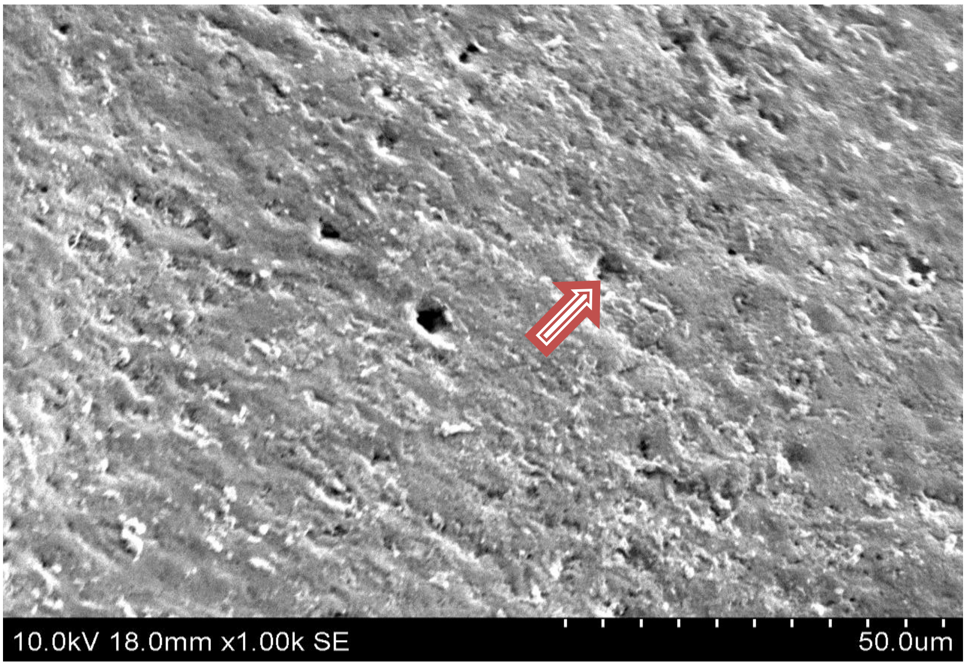
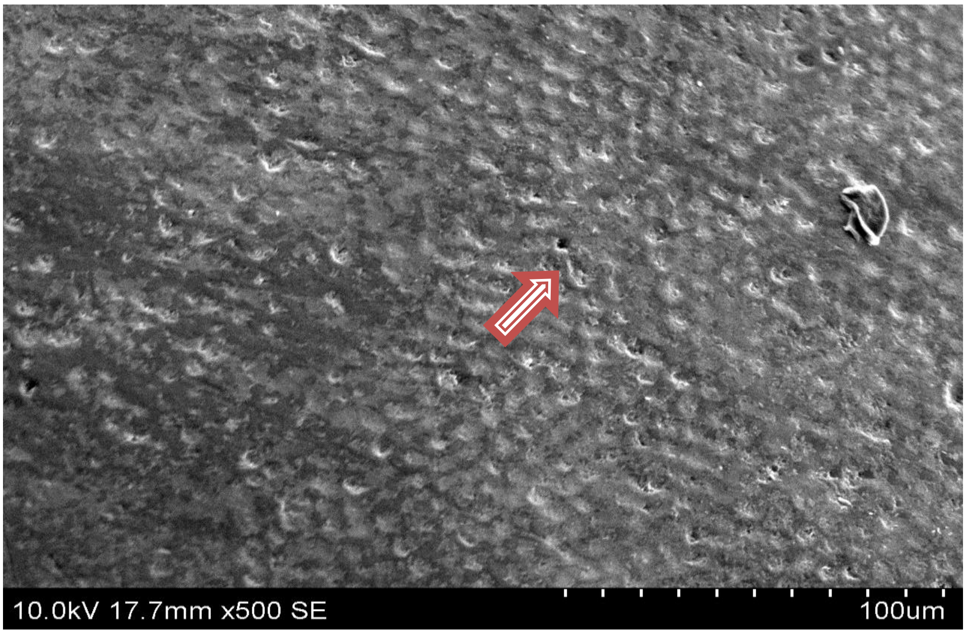


Fig 23: Acid challenge of artificial saliva remineralized enamel results in more irregular surfaces with severe porosities indicating less resistance to demineralization.

ACID CHALLENGE OF REMINERALIZED ENAMEL

Group II (CPP-ACFP):



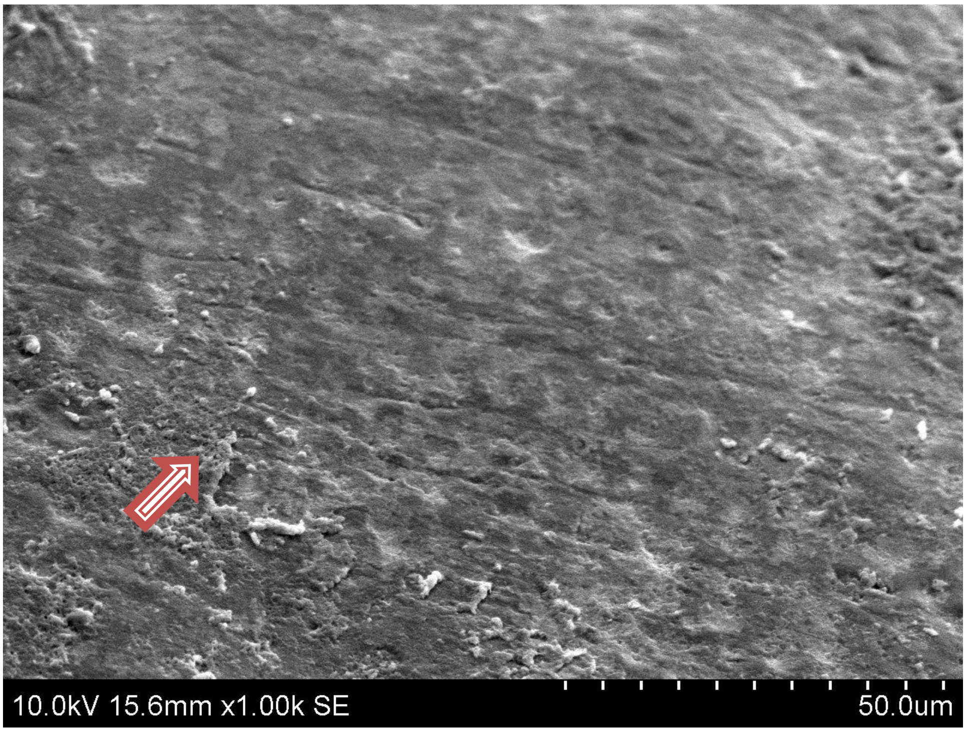


Fig 24: Acid challenge of CPP-ACFP remineralized enamel surfaces resulted in irregular surfaces with few porosities indicating moderate resistance to demineralization.

ACID CHALLENGE OF REMINERALIZED ENAMEL

Group III (fTCP):

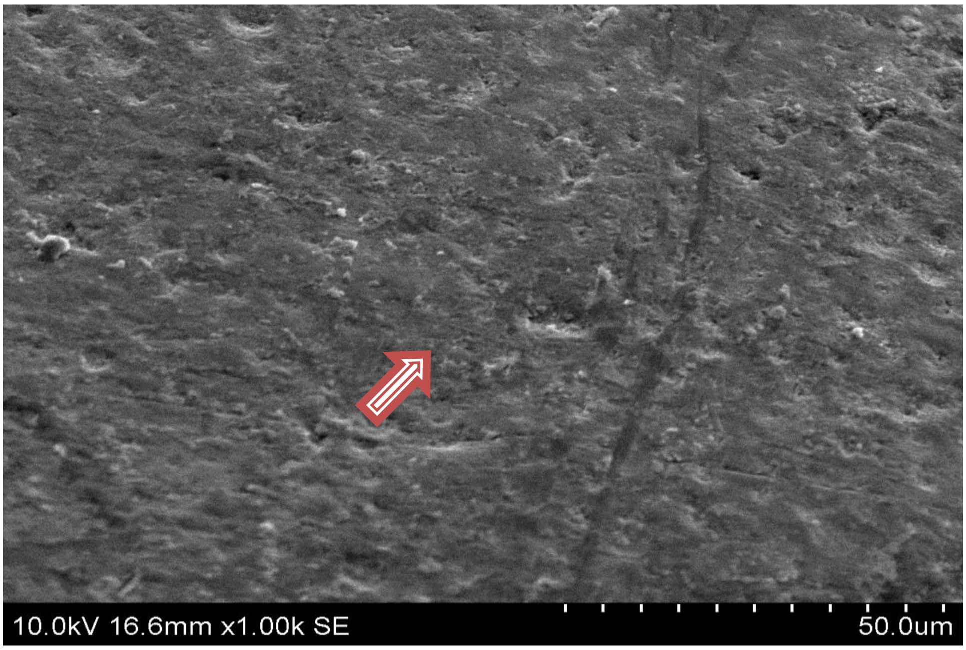


Fig 25: Acid challenge of fTCP remineralized enamel surfaces results in relatively smoother surfaces indicating greater resistance to demineralization.

DISCUSSION

Dental erosion is described solely as a surface phenomenon of tooth unlike dental caries which includes destructive effects on both the surface and the subsurface regions. Studies in vivo and in situ, suggest that, of the three tooth wear processes - attrition, abrasion and erosion, the most common threat for tooth surface loss is erosion.[83] Besides removal and softening of the surface, erosion may show dissolution of mineral contents underneath the surface.

The major extrinsic cause of dental erosion is excessive consumption of acidic beverages and it has considerably risen over recent years[18.] Erosion could involve two types of wear of enamel, first is the direct removal of hard tissue by complete dissolution and second is creation of a thin softened layer underneath the enamel, which is vulnerable to subsequent mechanical wear. The pH fall at tooth surfaces following a single ingestion of acidic beverages is shortlived and likely to produce softening on surface layer, but repeated intake of erosive drinks might favour more advanced demineralisation in sub surface layer resulting in total tissue loss.[52,9].

Exposure of tooth surfaces to saliva, fluoride and dietary products has been shown to be effective in the remineralization of eroded enamel. Once the erosive agent i.e.acidic beverage is neutralized or cleared from the tooth surface, precipitation and deposition of salivary calcium and phosphate can lead to remineralization of softened enamel.Various studies suggest saliva as the most important biological factor influencing dental erosion. Saliva helps in preventing erosion due to its ability to act, directly on the erosive agent itself by neutralizing, diluting, clearing, and buffering acids and plays a role in forming a protective membrane over the tooth surface, and providing calcium, phosphate and fluoride to eroded enamel and dentin to reduce the demineralization rate and enhance remineralization[76].

Erosion is influenced by biological variations within dental tissues. The flow rate, electrolyte composition, buffer capacity and protein composition of saliva varies from individual to individual which is resposible for inter-subject variation in erosive process.These variations also influence the rate at which saliva recovers from undersaturation following an acid challenge and also determines the remineralization potential [3,11]. Saliva is the source of the acquired pellicle, which reduces the amount of mineral loss in short-term erosion. However, during repeated acid exposure, the pellicle is removed except for the dense basal layer and its protective effect is lost.[26] Studies indicated that significant protective effect of pellicle is achieved after exposure of saliva to enamel for 1 hour.[77]

Softened enamel when exposed to saliva or to a remineralising solution for an adequate time has potential to regain mineral and thus re-acquire mechanical strength[4].Natural saliva and its synthetic substitutes (artificial saliva) reduce enamel mineral loss[3], enhance enamel rehardening and decrease erosive lesion depth in various in vitro and in vivo studies.The artificial saliva used in this present study was aimed at simulating natural saliva relevant for remineralization processes. Therefore, the pH-value of the saliva (6.2 to 7.4) was adjusted to natural salivary pH under stimulation conditions and thus, demonstrates improved salivary buffer capacity.

Hydroxyapatite crystals are formed in the oral environment from supersaturated state of calcium, phosphates, and fluoride ions[21].It is established from various studies that fluoride reduces demineralization and enhances remineralization of the enamel surface.[80] The efficacy of fluoride in the remineralization process is dependent upon different factors including its bioavailability and higher concentrations which is more effective.[74.]

Fluoride provides its beneficial effects by incorporating in tooth mineral as fluorapatite leading to the decreased solubility of the tooth enamel[12]. Fluoride-releasing materials may act as a fluoride reservoir and may increase the fluoride level in dental hard tissues, saliva and plaque. The precipitation of the calcium and phosphate ions from neutral or basic solutions forms Amorphous Calcium Phosphate (ACP) which ultimately form a final stable crystalline product called Hydroxyapatite (HAP). ACP is considered as a potential remineralizing agent in dental applications due to its relatively high solubility and its conversion to HAP in aqueous media. ACP releases supersaturating levels of Calcium and Phosphate ions surrounding erosive lesions and shifts the solution's thermodynamic driving forces towards the formation of apatite[7].

The cluster sequence of Casein phosphopeptides (CPP) containing -Ser(P)-Ser(P)-Ser(P)-Glu-Glu- have a remarkable ability to stabilize amorphous calcium phosphate (ACP) in a metastable solution. CPP binds to form nanoclusters of ACP, through the multiple phosphoseryl residues, preventing their growth to the critical size which is required for nucleation and phase transformation. The nanocomplexes of CPP–ACP are casein-derived peptides, in which ACP is stabilized by CPP.When these nanocomplexes are incorporated into the dental plaque and on the tooth surface, they act as calcium and phosphate reservoir[62].CPP–ACP has been suggested as remineralizing agent which reduces demineralization and promotes remineralization. It is well known fact that the localization of ACP at the tooth surface buffers the free calcium and phosphate ion activities and maintains a state of supersaturation that could depress demineralization and enhance remineralization of the enamel[62].

Karlinsey et al (2009)[34] reported a new prospective calcium system that is prepared by reacting the soluble tricalcium phosphate with a surfactant to form a **functionalized tri calcium phosphate** (fTCP). It uses tricalcium phosphate particles which have been ball milled along with sodium lauryl sulphate .This technology has been included in a tooth cream with sodium fluoride in different concentrations. These components naturally absorbed by teeth, therefore helps in preventing the initiation and further progression of demineralization and allowing remineralization to occur. There are very less studies in the literature for the functionalised tricalcium phosphate paste with respect to remineralization on eroded tooth enamel. Therefore this present in vitro study has evaluated the remineralization potential of CPP-ACFP and fTCP pastes and erosion resistance of tooth enamel by analyzing surface microhardness and surface topography by SEM.

The oral environment including saliva, plaque, and bacteria can affect the surface of teeth even for a short period of time.Therefore mature unerupted human third molar teeth which were not exposed to oral environment were selected for this study[8]. This study design required a sufficiently flat surface area of enamel slabs to allow microhardness measurements, thus the area subjected to erosion was not the original surface enamel.Flat and polished enamel slabs were used in this present study in an attempt to standardize and to remove the natural variations in enamel surface between teeth and also between different sites and types of tooth, which may result in different responses to acidic dissolution[1]. Polishing of enamel slabs were carried out using metallographic polisher which removed approximately 0.18mm of the tooth surface. **Craig et al (1958)**[14] found that inner enamel is of comparable hardness to the surface enamel.Hence polishing of enamel slabs will not significantly affect the microhardness measurements rather it produces more even pattern of erosion.

Surface micro hardness and Scanning Electron Microscopic (SEM) evaluation:

Surface micro hardness indentation provides a simple, rapid and non-destructive method in demineralization and remineralization studies. Indentation hardness testing with either Vickers or Knoop indenter have been used for the measurement of initial enamel hardness, enamel softening as an initial manifestation of the erosive process, as well as enamel hardening after remineralization[17]. Both indenters are suitable for hardness testing of non-metallic materials.

The load of 100 g was chosen for this study for hardness indentation because they created longer Vickers diagonals, which were recommended to prevent errors in optical measurment[81.] Changes in structure of tooth due to extrinsic factors have been investigated widely through Scanning electron microscope **(SEM).** This method requires proper preparation of specimen (sputtering) and examination conditions. **Duschner et al (1997)**[19] reported that SEM offers information on surface as well as subsurface layers of tooth enamel after in vitro fluoride treatments.

The hardness values obtained for enamel slabs in this study were in the range of **330.0 to 349.6 VHN**, which were in agreement with the studies by **Gaspersic** and **Reyes-Gasga et al** [23,24] and also correlates with the normal micro hardness of enamel **(322 to 353 VHN)**[44]. Microhardness values decreases from the outer enamel surface towards the dentinoenamel junction, which may explain the range of baseline values obtained.

On exposure to a cola drink for 8 min, **surface microhardness values decreased to 77%** of the baseline values in this study. These values were similar to the study by **Tantbirojin et al (2008)**[73] who reported a reduction to **70%** of baseline values after erosion of bovine enamel in a cola drink for 8 min. Study by **Wangkhantee et al (2006)** [79] using human enamel found a **63%** reduction in Vicker's microhardness after teeth were alternately immersed in artificial saliva and cola drink. Similar to this study**, Devlin et al (2006)**[17] measured the surface microhardness of human enamel exposed to Coca-Cola and found that the hardness decreased with the exposure of cola drink, and a partial recovery of hardness when enamel samples were exposed to artificial saliva.

In the present study, SEM images of intact enamel surfaces show **relatively smooth surfaces** before erosion attack (Fig.20) while enamel surfaces after immersing in cola drink for 8 min resulted in **irregular surfaces with porosities** indicating the area of demineralization (Fig.21). As erosion is correlated to pH; the beverage with the lowest pH, Coca cola (2.34), was chosen for this study for erosive process and was replenished every 2 minutes for 8 minutes in order to stress their demineralization potential to ensure that it was carbonated and also to reduce the buffering effect from ions dissolved from the enamel surface.This maintained the tooth enamel in the initial stages of the erosive process, without reaching the level of visible surface loss.[73]

Following demineralization, Group I (**Control**) enamel slabs were stored in artificial saliva and did not receive any treatment. Group 2 enamel slabs were treated with **CPP-ACFP** paste with a cotton tip applicator for **3 min** at **0, 8, 24, 36 hrs** and Group 3 enamel slabs were treated with **fTCP** paste with a cotton tip applicator for **3**

min at **0, 8, 24, 36 hrs**[73].These application times were similar to the study by **Tantbirojin et al** who treated enamel slabs with **CPP-ACP** paste for 3 min at **0, 8, 24, 36 hrs**. Both the CPP-ACFP and fTCP pastes resulted in statistically significant increase in surface microhardness values from the demineralized values and comparable to the control group (artificial saliva).

In this present study, the **increase in microhardness values for control group was 7%** from its demineralized microhardness values while **increase in microhardness values for CPP-ACFP and fTCP groups were found to be 14.70%** and **22.1%** respectively from there demineralized microhardness values. In SEM evaluation, remineralized enamel slabs of control group showed reduction in some porosity with **inadequate remineralization** (Fig.22) CPP-ACFP group showed **adequate remineralization** with reduction in porosities (Fig.23) and fTCP group with **enhanced remineralization** throughout the surfaces with more homogenous morphology (Fig.24).

These results of this study were consistent with the results found by **Karlinsey et al (2009)**[34] that they compared Functionalised tricalcium phosphate with MI paste in terms of remineralization and observed that the combination of functionalized tricalcium phosphate and NaF at different fluoride levels provides a successful dose response remineralizing effect. **Karlinsey et al (2008)**[32] also concluded that 1,000 ppm fluoride fTCP dentifrice showed fluoride uptakes and mean Vickers hardness recoveries higher than those of the paste with CPP-ACP and 900 ppm fluoride in remineralization of white-spot enamel lesions.

A study done by **Poggio et al (2009)** showed that CPP–ACP increases microhardness of enamel eroded by cola drink implies that its erosion inhibiting potential might involve remineralization action**. Cross et al (2004)**[16] suggested that CPP also stabilizes Amorphous calcium fluoride phosphate (ACFP) which has greater remineralizing effect as compared to fluoride or CPP –ACP individually on carious lesions.The possible explanation for remineralizing potential of CPP-ACP and fluoride combination is co-localization of calcium and phosphate ions along with fluoride ions at the eroded enamel surface, presumably as CPP-ACFP nanocomplexes.Recent study by **Srinivasan et al (2010)**[72] showed that CPP–ACP exhibited a higher remineralizing potential when combined with 900 ppm of fluoride than CPP ACP used alone.

Acid challenge of remineralized enamel slabs with cola drink for 8 min resulted in demineralization.**16%** reduction of microhardness values from its remineralized values were noted for **Control group** and reduction in microhardness values of **11.60% and 7.22%** were noted for CPP-ACFP and fTCP groups' respectively.This residual hardness after acid challenge was significantly greater for fTCP than CPP-ACFP.

The SEM images also confirmed the relatively lower solubility of the remineralized zone of enamel surfaces after remineralization with fTCP (fig:27) indicating greater resistance to demineralization while CPP-ACFP showed irregular surfaces with few porosities(fig:26) indicating moderate resistance to acid challenge and control group showed erosive pattern throughout the surfaces indicating least resistance to acid challenge(fig:25)

The morphological interpretation of SEM images suggested that fTCP showed the greatest amount of remineralization followed by CPP-ACFP and artificial saliva.Also the protective effect through acid challenge is considered more evident for fTCP paste. **Ijima et al (2004)**[29] concluded that remineralized enamel lesions through application of remineralizing agents were more resistant to subsequent acid challenge.

The reason for the enhanced remineralizing potential of fTCP when compared to CPP-ACFP as stated by manufacturer is that during manufacturing of the toothpaste, a protective barrier is created around the calcium ions thereby allowing it to coexist with the fluoride ions. During brushing when the toothpaste comes into contact with saliva, the barrier dissolves and allows the release of calcium, phosphate and fluoride ions on the tooth surfaces to help prevent tooth decay and remineralize demineralized enamel. The exclusive manufacturing of fTCP in terms of milling process protects the TCP during storage so that the calcium does not degrade the fluoride.Various organic materials can be used to tailor the fTCP system to a different topically applied oral care preparations, such as toothpaste, oral rinses and varnishes.

Within the limitations of this study, the fTCP and CPP-ACFP pastes are delivery vehicles to localize fluoride, calcium, and phosphate at the tooth surfaces. The findings of this present in vitro study suggested that **fTCP with 950 ppm of fluoride resulted in enhanced remineralization compared to CPP-ACFP with 900 ppm of fluoride**.However further long term and in vivo studies need to be done to ensure these results.

Summary

This study was undertaken to compare the evaluation of **CPP-ACFP** and **functionalised tricalcium phosphate** (fTCP) pastes on remineralization and erosion resistance of remineralized tooth enamel.

For evaluating the Surface Microhardness and Scanning Electron Microscope (SEM),15 unerupted, matured human third molar teeth were used.Out of these, 60 enamel slabs were made and baseline surface microhardness measurements were carried out.From these,45 enamel slabs were selected with microhardness values in a range of 339.0-349.6 VHN and followed by SEM analysis.After the baseline microhardness and SEM analysis, enamel slabs were immersed in cola drink for 8 minutes at room temperature followed by microhardness measurement and SEM analysis.Then these softened enamel slabs were randomly divided into 3 groups as follows

Group I- Control (n=15)

Group II- CPP-ACFP (n=15)

Group III – fTCP (n=15)

Group I received no treatment and was stored in artificial saliva for 36 hrs while Group II and Group III were treated with CPP-ACFP and fTCP pastes respectively for 3 min at 0, 8, 24, 36 hrs.All enamel slabs were taken for assessment of surface microhardness changes and SEM evaluation.

Acid challenge of remineralized enamel slabs were carried out by immersing in cola drink for 8 min followed by assessment of surface microhardness changes and SEM evaluation.Statistical analysis was performed using one way

ANOVA test followed by Post-hoc multiple comparisons by Tukey's HSD test at a significance level of 0.05.

Results of the microhardness measurement of the enamel slabs showed that both Group II (CPP-ACFP) and Group III (fTCP) exihibited statistically higher remineralizing potential than Group I (Control) while Group III had statistically significant remineralizing potential than Group II.

Results of the SEM analysis showed that Group III (fTCP) resulted in enhanced remineralization throughout the surfaces, with a more homogenous morphology while Group II (CPP-ACFP) resulted in adequate remineralization with reduction in porosities and Group I (control) resulted in inadequate remineralization. Acid challenge of Group III (fTCP) remineralized enamel slabs resulted in relatively smoother surfaces, indicating greater resistance to demineralization, Group II (CPP-ACFP) resulted in moderate resistance to demineralization and Group I (Control) resulted in more irregular surfaces with severe porosities indicating less resistance to demineralization.

Conclusion

Within the limitations of this in vitro study the following conclusions could be drawn:

- fTCP showed higher remineralizing potential than CPP-ACFP
- In addition, fTCP showed superior acid resistance compared to CPP-ACFP.
- It can be recommended that fTCP be used as topical tooth crème.
- Further in vivo studies are required to confirm the beneficial effects of fTCP over CPP-ACFP.

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